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Induction of a refractory state to viral infection in mammalian cells by a plant inhibitor isolated from leaves of *Melia azedarach* L

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Summary

A partially purified plant inhibitor (Meliacin) isolated from *Melia azedarach* L induced in cells a refractory state to virus infection. Meliacin was active in a large variety of continuous and/or primary cell cultures. A state of maximum virus resistance was achieved after 2 h of incubation and was maintained for at least 15 h; later on it declined but it was fully regained after a second pulse of Meliacin. Interferon was not detected in the supernatant of cells treated with Meliacin and a measurable increase in ds-RNA dependent protein kinase activity was not observed in extracts of Meliacin-treated cells. The antiviral state was not transferred by either extracellular fluid or direct cell-to-cell contact. An active cell metabolism was required for Meliacin action, which was partially reversed in the presence of actinomycin D. It appears that Meliacin is not an interferon-like substance, which induces an antiviral state based on a still unexplained mechanism.

Melia azedarach L; Antiviral state; Plant antiviral

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Introduction

Root or leaf extracts from *Melia azedarach* L inhibit the in-vitro replication of various DNA and RNA animal viruses (i.e. vesicular stomatitis virus, herpes simplex type I, poliovirus, Sindbis, Junín, Tacaribe and Pichinde) (Andrei et al., 1985; Wachsman et al., 1982). The partially purified inhibitor (Meliacin) also displays a broad spectrum of antiviral activity without any toxic effect on cultured cells (Andrei et al., 1985). In addition, Meliacin treatment prevents the development of encephalitis caused by Tacaribe virus in neonatal mice (Andrei et al., 1986). Very recently we succeeded in the identification of a glycopeptide (MW: 5–6 kDa) which was found to be responsible for the antiviral activitiy of crude extracts from *M. azedarach* L (Andrei et al., to be published elsewhere).

In the presence of Meliacin, virus multiplication is arrested, but Meliacin is also able to trigger an antiviral state by cell pretreatment prior to infection (Wachsman et al., 1982). Therefore, the possibility of Meliacin acting as an interferon-like substance or through induction of interferon or other antiviral cellular molecules has to be considered.

In order to clarify the molecular mechanism leading to viral resistance in Meliacin treated cells, we have directly examined whether the observed antiviral effect is interferon-mediated or comprises a different mode of action.

Materials and Methods

Cells

HEp-2, HeLa, Vero and L-929 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5% inactivated calf serum and 50 μg/ml gentamycin. BHK-21 and RK-13 cells were grown in Dulbecco's modified MEM supplemented with 8% calf serum and antibiotics. Confluent 48 h old monolayers were used throughout all the experiments. Maintenance medium consisted of Eagle's basal medium (BME) containing 2–3% calf serum and gentamycin.

Primary cell cultures from chicken embryo (CE) were obtained by standard procedures; they were grown in MEM supplemented with 10% calf serum and were maintained in MEM with 5% calf serum.

Viruses

Vesicular stomatitis virus (VSV), Indiana serotype, was grown in BHK-21 cells. Virus stocks were prepared from culture media of cell monolayers infected 20–24 h before at an input multiplicity of infection (MOI) of 1 PFU/cell. Newcastle disease virus (NDV), La Sota strain, was propagated in 11-day-old fertile hen eggs and had a titer of 1.2×10^4 HA-U (hemagglutinating units)/ml.

Meliacin preparation and purification

Melia azedarach L fresh green leaves were used as source of Meliacin. The specimens, collected in Buenos Aires city, were identified and are on deposit at the

Department of Botany Science School, Buenos Aires University (Argentina BAFC 1432). The plant material was washed with distilled water and stored at −20°C until use. Routinely, the thawed material was cut into small pieces and ground in a Waring blendor with an equal weight of 10 mM potassium phosphate buffer, pH 7.2 (PK buffer) containing 0.35 M KCl. The sap obtained was pressed through cheesecloth and was centrifuged at 10000 × *g* for 1 h. The supernatant fluid was separated and dialysed overnight with mechanical stirring against 100 volumes of PK buffer. All operations were carried out at 4°C. The non-dialyzable fraction was centrifuged at 10000 × *g* for 20 min to remove turbid material. Aliquots of this fraction were concentrated by lyophilization and stored at −20°C. The powder thus obtained was resuspended in distilled water to concentrate the material 10-fold. Samples were clarified by centrifuging at 10000 × *g* for 20 min and the supernatant fluid was chromatographied on a Sephadex G-100 column. Most of the protein was bound to the column, whereas the inhibitory activity appeared in a single peak in the break-through volume. Those fractions which were found to contain the antiviral activity were combined and applied on a DEAE-Sephadex column equilibrated with PK buffer. Then, the column was washed with buffer to remove unadsorbed or weakly adsorbed components, resulting in the antiviral factor appearing as a peak in the effluent. The fractions containing the inhibitor were pooled and used as source of Meliacin. Stocks thus obtained contained approximately 80 antiviral units/ml (One antiviral unit/ml of Meliacin is defined as the inverse of the dilution that reduced plaque formation of VSV in Vero cells by 50%).

Demonstration and quantification of the antiviral state

Cell monolayers were pretreated with BME or BME containing Meliacin at 37°C for the indicated time. Then, media were removed and the cultures were washed twice with PBS and infected with VSV at a MOI of 1. After incubation at 37°C for 1 h to allow virus adsorption, the inocula were removed and therefore infected cells were incubated with BME alone. Supernatants were harvested at 16–20 h post-infection (p.i.) and virus was titrated by plaque assay. The ratio between virus titer in Meliacin treated cultures and virus titer in untreated cell cultures (V_t/V_o) represents the reduction in virus yield.

Transfer of antiviral resistance assay

Vero or BHK-21 cells were incubated with medium or medium plus Meliacin for 2 h at 37°C. Then, the monolayers were trypsinized and resuspended in MEM 5% calf serum. Meliacin-treated or control donor cells were added to a new set of untreated recipient cell monolayers and were cocultured at a 1:1 ratio. The total number of cells/culture was 1.0×10^6 in 1 ml, which comprised 5.0×10^5 cells/culture of each cell type. Controls consisted of an equivalent number of cells alone. After 2 h incubation at 37°C the supernatant fluids were decanted and each cell monolayer was infected with VSV at a MOI of 1. Virus yields from pooled duplicate cultures were determined approximately 18 h later by plaque assay. The $\log_{10}$ inhibition of VSV yield per cell was calculated by the formula:

$$-\log_{10} \frac{\text{PFU/cell from cell mix with Meliacin treated donor cells.}}{\text{1/2 PFU/cell from cell mix with untreated donor cells.}}$$

Interferon induction

Murine interferon was induced by treating confluent 2 days old L-929 cell c tures with 320 HA-U of NDV for 1 h. Then, cultures were washed and reple ished with maintenance medium. Interferon preparations obtained after differe times p.i. from NDV-induced cultures were acidified to pH 2 for 5 days and th adjusted again to pH 7.4.

The potential capacity of Meliacin as an interferon inducer was tested as f lows: confluent cultures of L-929 cells were incubated with Meliacin for 2 h at 37° followed by washing and addition of fresh medium. Supernatants were collect at variable times after induction and assayed for interferon activity after acid tre ment.

Interferon assay

L-929 cells were incubated for 24 h after addition of serial two fold dilutions the above samples. Then, they were washed twice with PBS and infected with 1(150 PFU of VSV. The monolayers were incubated at 37°C for 48 h, stained, a plaques were counted. The interferon titers are expressed as the reciprocal of t dilution of sample which reduced the number of plaques by 50%.

Protein-kinase assay

Confluent L-929 cells were treated at 37°C for 24 h with 500 IU/ml of mou fibroblast interferon (Sigma), Meliacin (1:5 dilution) or BME. After incubatic cell extracts were prepared as described by Kimchi et al. (1979). Protein-kina activity was determined as described by Moreno et al. (1977) with slight mod cations. Aliquots of 50 µl of cell extracts containing approximately 125 µg of p tein were preincubated in 90 µl of buffer B (Moreno et al., 1977), 0.5 µg/ml p (rI), poly (rC) (Sigma) and 0.5 mM ATP for 15 min at 30°C. Then, 10 µl of stone (type IIA, Sigma) 1.5 mg/ml and 0.1 mM (γ $-^{32}$P) ATP (400 cpm/pmol) w added. After incubation at 30°C for 30 min samples were processed as describ by Moreno et al. (1977). Enzyme activity was expressed as picomoles of ^{32}P bou in 30 min per mg of protein under the standard assay.

Results

Host-range of Meliacin action

Since VSV can be propagated in a broad spectrum of vertebrate cells, it v possible to analyse the ability of Meliacin to induce an antiviral state in cells fr different species. Meliacin triggered a refractory state to VSV multiplication in cells tested, although quantitative differences were noted (Table 1). As Vero c were very susceptible to Meliacin action, we chose this cell line for further exp iments.

TABLE 1

Inhibition of VSV replication in different host cells pretreated with Meliacin

Cells	VSV yields (PFU/ml)		
	Control	Treated	$-\log V_t/V_o$
Vero	2.2×10^7	1.7×10^4	3.11
BHK-21	1.0×10^7	2.1×10^5	1.67
Hep-2	4.0×10^6	8.0×10^5	1.70
HeLa	3.0×10^7	5.5×10^4	2.73
CE	2.5×10^4	1.0×10^1	3.39
RK-13	4.5×10^7	5.5×10^5	1.91
L-929	3.8×10^6	1.0×10^4	2.57

Cell cultures were exposed to BME or BME plus Meliacin (1:5 dilution) for 2 h at 37°C. Then media were removed and the cultures were washed and infected with VSV at a MOI of 1. At 18 h p.i. virus yields were determined by plaque assay in the same cell system as used for virus growth.

The effect of Meliacin added at different times after cell seeding was also investigated. Although Meliacin was active on both young and aged cultures (Fig. 1), it can be seen that VSV replication was less efficient in older cells.

Kinetics of induction of the antiviral state

To determine the kinetics of establishment of the refractory state, monolayers of Vero cells were pretreated with BME or with different dilutions of Meliacin for various periods of time. Each culture was then washed and challenged with VSV at a MOI of 1, and virus yields were determined at 20 h p.i.

Results plotted in Fig. 2A show that the establishment of the antiviral state is

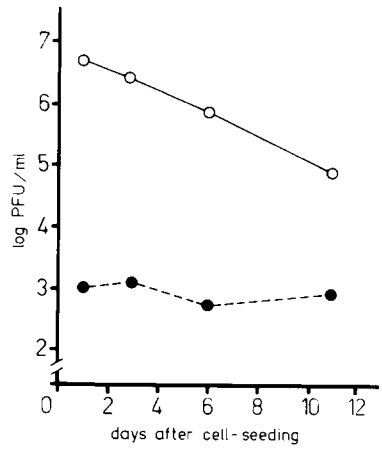

Fig. 1. Meliacin induced anti-VSV resistance in Vero cells at different states of cell growth. Monolayers obtained after 1, 3, 6, 9 or 11 days of cell-seeding were incubated in the presence or absence of Meliacin (1:5 dilution) for 2 h at 37°C. Then treated (-●-●-) and untreated control (-○-○-) cultures were washed and challenged with VSV. Virus yields were estimated by plaque assay at 20 h p.i.

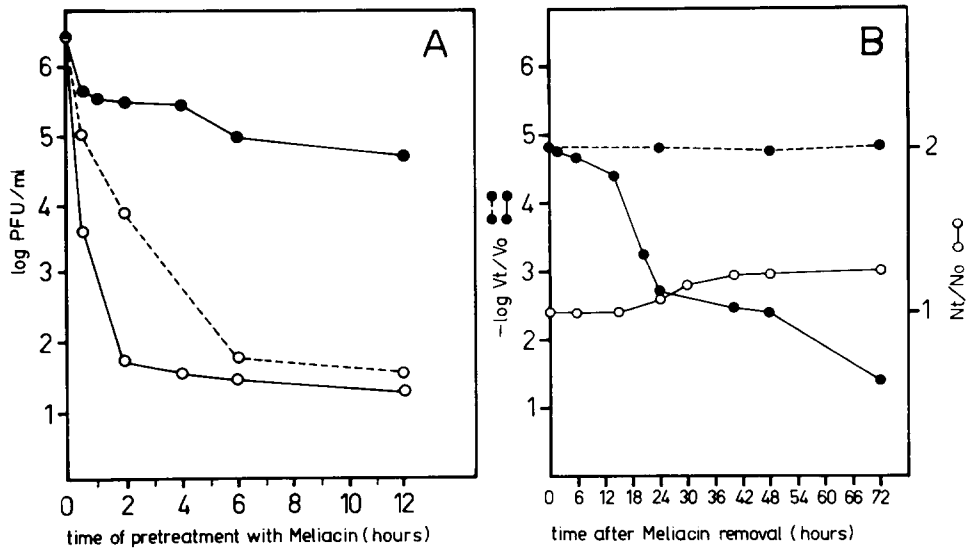

Fig. 2. (A) Kinetics of establishment of the antiviral state. Vero cell cultures were incubated with or without 1:10 (-○-○-), 1:20 (--○--○--) or 1:40 (-●-●-) dilutions of Meliacin for different times at 37°C. Then the monolayers were washed and infected with VSV at a MOI of 1. After adsorption for 1 h at 37°C the inocula were discarded and virus yields were determined at 20 h p.i. (B) Duration and reinduction of the antiviral state. Vero cells were exposed to BME or 1:5 dilution of Meliacin for 2 h at 37°C ("zero time"), followed by washing and addition of fresh media and incubation at 37°C. A set of control and Meliacin-treated cultures were infected with VSV at varying periods of time after Meliacin removal (-●-●-), while another one was subjected to a second 2 h pulse of medium or Meliacin at 24, 48 and 72 h after Meliacin first pretreatment and immediately challenged with VSV (--●--●--). In all cases virus yields were determined by plaque assay at 20 h p.i., V_t/V_o being the ratio between virus titer in Meliacin-treated cultures and virus titer in control ones. A set of cultures were run in parallel to quantitate the number of viable cells by trypan blue exclusion at the moment of infection, N_t/N_o being the ratio between the number of cells at "t" time and the number of cells at "zero" time (-○-○-).

Meliacin-concentration dependent. Maximal inhibition is achieved with 1:10 and 1:20 Meliacin dilutions but the time necessary to reach the maximum refractory state was 2 and 6 h, respectively. Considering these results, we selected 2 h of cell treatment with the highest concentration of Meliacin as standard assay. Under these conditions, the kinetics pattern was the same in all cells tested (data no shown).

Duration of the antiviral state

Vero cells were incubated with BME or Meliacin for 2 h and then washed. Meliacin-treated and control cultures were infected with VSV at different times after Meliacin removal and virus yields were determined at 20 h p.i.

Fig. 2B illustrates that Vero cells were fully protected up to approximately 12 h after Meliacin removal. Between 12 and 24 h, a rapid decay occurred, and from 30 to 54 h a steady-state was reached. However, a residual effect of Meliacin at 72 h was still detected.

The rate of cell growth, also shown in Fig. 2B, could not account for the decay of the antiviral state, since the number of cells per culture varied from 2.25×10^5 (zero time) to 2.7×10^5 at 72 h p.i.

Then, the question was raised whether the antiviral state could be regained by a second exposure of cells to Meliacin. As Fig. 2B shows, after a second pulse of Meliacin, Vero cells became again fully resistant.

Requirement of a metabolic cell-process

To determine if Meliacin requires an active cell metabolism to trigger the antiviral state, monolayers of Vero or BHK-21 cells were exposed to medium or medium plus Meliacin for 2 h at 37°C or 4°C, followed by washing and challenge with VSV. The data of Table 2 show that cells treated at 4°C were not resistant to VSV replication indicating a requirement of an active cell metabolism for Meliacin action.

To investigate whether the induction of a cellular RNA or protein is involved in the antiviral action of Meliacin, its effect on VSV replication was determined in the presence of actinomycin D. For that purpose, actinomycin D (5 μg/ml) was added to Vero cells 1 h prior to Meliacin, simultaneously or at different times post-Meliacin treatment. This drug concentration inhibited cellular RNA transcription more than 99%, while VSV RNA synthesis was not affected (data not shown). Fig. 3 shows that maximal reversion occurred when actinomycin D was added 1 h before or simultaneously with Meliacin; later on this effect was rapidly lost and once established (at 2 h post-Meliacin treatment) actinomycin D had no longer any effect.

Absence of extracellular mediators in the mechanism of antiviral activity

As suggested from actinomycin D experiments it seemed likely that Meliacin induced the synthesis of an antiviral protein that could eventually be released. Thus,

TABLE 2

Influence of temperature on the establishment of the antiviral state

Cells	Cell-pretreatment		VSV yields (PFU/ml)	$-\log V_f/V_o$
	Meliacin	Temperature (°C)		
Vero	−	37	1.8×10^7	−
	+	37	5.0×10^4	2.55
	−	4	1.6×10^7	−
	+	4	1.0×10^7	0.20
BHK-21	−	37	9.5×10^7	−
	+	37	1.2×10^6	1.89
	−	4	8.6×10^7	−
	+	4	8.0×10^7	0.03

Vero or BHK-21 cells were pretreated during 2 h at 37°C or 4°C with medium or a 1:10 dilution of Meliacin, followed by washing and infection with VSV at a MOI of 1. At 20 h p.i. media were harvested and titrated for infectivity.

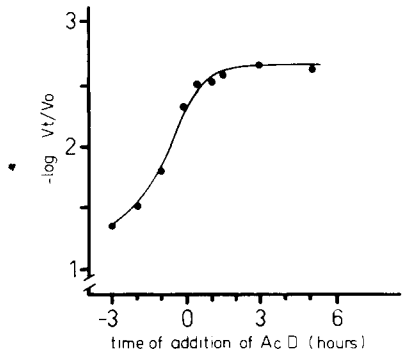

Fig. 3. Kinetics of reversion of Meliacin activity by actinomycin D. Vero cells were exposed to Meliacin (1:10 dilution) for 2 h at 37°C (−2 h to 0 h). After that period of time, residual Meliacin was completely removed by washing and the monolayers were then infected with VSV (O h). An equal number of control cultures were treated in exactly the same way except that they were not exposed to Meliacin. At different times before, during and after Meliacin cell-treatment, actinomycin D (5 μg/ml) was added to each culture and in all cases the inhibitor was present until the time of virus harvest at 9 h p.i. Virus yields were determined by plaque assay.

attempts were made to demonstrate antiviral activity in the cell culture fluid of Meliacin-treated cells. Vero or BHK-21 cell monolayers were incubated with or without Meliacin for 2 h at 37°C, then cultures were washed and BME alone was added. At 4, 24 and 36 h after Meliacin removal, supernatants from control and treated-cultures were harvested and added to a new set of monolayers and after incubation for 24 h at 37°C the cells were washed and challenged with VSV. Virus yields from cultures incubated with supernatants from Meliacin-treated or untreated cells were similar (data not shown).

The effect of actinomycin D on the antiviral state induced by Meliacin indicated that one or more proteins might be involved in the development of this refractory state. Although no antiviral activity was detected in supernatants of Meliacin-treated cells, possibly resistance could be transferred between cells by cocultivation. To demonstrate this we performed experiments similar to those described by J.R. Blalock (1981), who was able to show cell-to-cell transfer of virus resistance induced by interferon.

Cell mixtures were used at confluency, using a 1:1 ratio of Meliacin-treated to untreated cells or equivalent mixtures of untreated cells. Then cocultures were infected with VSV and the log inhibition of virus yield/cell was taken as a measure of the transfer of Meliacin-induced virus resistance. Values obtained ranged from 0.006 to 0.2, indicating that there was no transfer of resistance from Meliacin-treated to untreated cells in any cell-mixture combination.

All these data strongly suggest that Meliacin acts by a mechanism independent of interferon induction. However, since all these experiments were performed with Vero or BHK-21 cells, which are not good interferon producers, L-929 cells were selected for further experiments.

The ability of Meliacin to induce interferon in L-929 cells was investigated. At 2, 7, 24 and 48 h after Meliacin treatment, L-929 cell supernatants were collected and assayed for the presence of interferon activity. In parallel, supernatants of L-929 cells induced with NDV were tested. Whereas cells induced with NDV produced high titers of interferon, L-929 cells treated with Meliacin did not produce an extracellular interferon like-inhibitor at any time assayed (data not shown).

Despite these negative results we investigated whether one of the enzymes known to be induced by interferon was produced in response to Meliacin. The activity of ds-RNA dependent protein-kinase in Meliacin treated cells was compared to that found in interferon-treated cells. As shown in Table 3, no measurable increase in ds-RNA dependent protein-kinase activity was observed in extracts derived from Meliacin-treated L-929 cells. In fact, ds-RNA independent phosphorylation was reduced in Meliacin-treated cell extracts as compared to control cell extracts. In interferon-treated cells, the endogenous protein kinase activity was slightly increased in the presence of ds-RNA and it was doubled when exogenous histone was added.

Discussion

The experiments described here demonstrated that *Melia azedarach* L leaves contain a virus inhibitor able to trigger an antiviral state in cells by a mechanism independent of interferon.

A relationship between the antiviral action of interferon and Meliacin is suggested by results reported previously (Wachsman et al., 1987) and new data presented here. Thus, Meliacin's broad spectrum of antiviral activity (Andrei et al., 1985; Wachsman et al., 1982), its ability to induce an antiviral state in many different cell types (Table 1) and the fact that it does not inactivate virus (Wachsman et al., 1987, 1982), are properties very much alike those described for interferons (De Maeyer and De Maeyer-Guignard, 1979; Ho and Armstrong, 1975; Lengyel, 1982).

TABLE 3

Protein kinase activity in cell extracts from Meliacin-treated and untreated cells

Cells preincubated with	ds-RNA	Protein kinase activity[a]	
		Endogenous phosphorylation	Exogenous phosphorylation (+ histone)
BME	−	56.1	99.6
BME	+	44.9	118.4
IFN	−	40.8	130.1
IFN	+	68.8	270.5
Meliacin	−	21.5	50.4
Meliacin	+	24.5	51.2

[a]Protein kinase activity is expressed as picomoles ^{31}P per mg of protein incorporated in 30 min.

Despite these observations, our data exclude the possibility that the classical interferons were induced in response to Meliacin pretreatment. No detectable interferon activity was found in supernatants of Meliacin-treated Vero or BHK-21 cells. Likewise, L-929 cells, which produced high levels of interferon after NDV induction, did not release an extracellular inhibitor after Meliacin treatment. Also, we failed to transfer viral resistance by cell-to-cell contact as it was demonstrated to occur in interferon-treated cells (Blalock, 1981). All these data indicate that participation of interferon in Meliacin's antiviral activity is unlikely. This assessment was confirmed by measuring protein-kinase activity dependent on ds-RNA. As expected, interferon pretreatment of the cells yielded a clear increase of this activity, while no increase was detected in Meliacin-treated cells (Table 3). Curiously, a yet unexplained decrease in ds-RNA independent phosphorylation was apparent in extracts from Meliacin-preincubated cultures in comparison with normal cells. Whether the inhibition of phosphorylation by Meliacin reflects an in-vivo toxic effect of the compound remains to be elucidated.

If there is no relationship between the antiviral action of Meliacin and interferon, which are the molecular events leading to viral resistance?

We are not certain whether Meliacin is bound to the cellular surface or whether it enters the cell. In either case, metabolic activity at 37°C is required for antiviral activity to become apparent, because the antiviral state induced by Meliacin is not established at 4°C, but requires incubation at physiological temperature (Table 2). Experiments reported in Table 2 and Fig. 3 indicate that cellular proteins might probably participate in virus resistance, since the antiviral effect of Meliacin is partially reversed when cellular RNA synthesis is completely blocked by actinomycin D.

Several compounds of vegetal origin have been reported to act as interferon-like substances (Chessin, 1983). However, their mode of action was not thoroughly investigated. As shown by our results, it appears that Meliacin is not an interferon-inducer-like compound. It activates the cell to develop an antiviral state by a complete novel mechanism, which remains open for investigation.

Acknowledgements

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