Dependence of the drift of the erythrocyte-flow line on the electronic state of hemoglobin[a]

	Oxy	CN-met	Deoxy	Met
Drift (μm/s)	0	0.38	2.0	2.6
M (μ_B)	0	2.2	5.3	5.8
M^2	0	4.8	28.1	33.6

[a] M represents the magnetic moment of hemoglobin in units of Bohr magneton(μ_B)[2,3,7]. Erythrocytes containing oxygenated hemoglobin (Oxy) were obtained by washing and suspending the erythrocytes in a isotonic buffered saline (pH 7.7). Erythrocytes containing deoxygenated hemoglobin (Deoxy) were prepared by suspending the washed erythrocytes in the solution of 25 mM sodium hydrosulfite. In the experiment with deoxygenated erythrocytes, all the solutions in the flow apparatus were deaerated by bubbling nitrogen gas. The washed erythrocytes were treated with $NaNO_2$ (20 mM) to oxidize hemoglobin and washed five times and then suspended in 50 mM phosphate buffered saline of pH 5.7 to retain the electronic configuration in the high spin state (Met). Erythrocytes containing cyano-methemoglobin (CN-met) were prepared by incubating the washed erythrocytes containing methemoglobin, with KCN (10 equivalents of the total heme) and washed three times before suspending in the buffered saline of pH 7.4. The diameter of the flow line was about 80 μ.

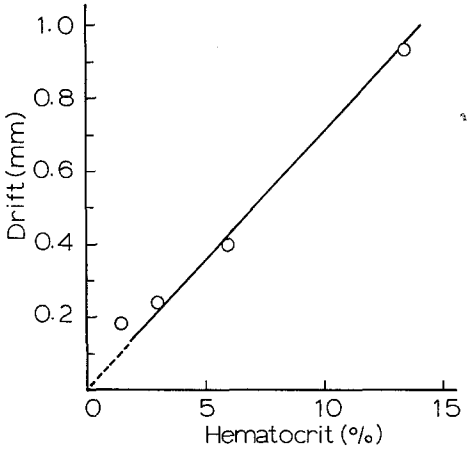

Figure 2. Dependence of the drift of the erythrocyte-flow line on the hematocrit value. The erythrocytes containing high spin methemoglobin was allowed to flow during 150 s in the magnetic field whose averaged strength and gradient were 0.6 T and 48 T/m, respectively. The hematocrit value of the flowing erythrocyte suspension was obtained as follows: (1) The erythrocyte suspension, which flowed out from the observing cell, was collected, and the density of erythrocytes was determined by counting the erythrocytes in it. The volume fraction of the erythrocyte flow in the bulk flow of the buffer was calculated using the diameter of the erythrocyte flow (circa 80 μm) and the theoretical velocity distribution in a rectangular cell (of infinite width).

ity of the drift to the hematocrit value indicates that the drift is very much amplified by the hydrodynamic interaction among the erythrocytes. This fact may imply that a certain volume of the erythrocyte suspension is attracted as a whole by the force represented by Eq. 1; in this case V and χ represent the volume and the magnetic susceptibility of the mass of the flowing erythrocyte suspension. This volume may be approximated as a sphere whose diameter is that of the erythrocyte-flow line. If 50 μm for R and 0.06 χ (the magnetic susceptibility of the erythrocyte) for the susceptibility are adopted for the suspension of 6% hematocrit, we obtain 2.0 μm/s for the drift velocity, which is in good agreement with the observed value.

In the actual flow line, there may be a little inhomogeneity in the distribution of erythrocytes. In addition, a flow like an eddy current of the flowing buffer, due to the drift of the erythrocyte flow, may occur. These factors may cause the broadening of the erythrocyte-flow line.

The physiological effects of this phenomenon, such as drift and broadening of the erythrocyte flow caused by the magnetic field, are not clear at the present stage. However, we would suggest that the rheological properties of erythrocytes in the venous system might be influenced by the inhomogeneous magnetic field.

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An antiviral factor from *Melia azedarach* L. prevents Tacaribe virus encephalitis in mice[1,2]

G.M. Andrei, J.S. Lampuri, C.E. Coto[3] and R.A. de Torres

Laboratorio de Virología, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, piso 4, Ciudad Universitaria, 1428 Buenos Aires (Argentina), and Departamento de Microbiología e Inmunología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, 1113 Buenos Aires (Argentina), 19 September 1985

Summary. Treatment of neonatal mice with an antiviral factor, (AVF), obtained from the leaves of *Melia azederach* L. protected them against lethal encephalitis caused by Tacaribe virus inoculation. The degree of protection obtained varied from 66% to 100% depending on the virus dose. Similarly, administration of AVF to nursing mothers protected their offspring from developing virus encephalitis. AVF does not directly inactivate Tacaribe virus; it inhibits an early step (s) in the replication process in cell cultures.
Key words. Antiviral activity; Tacaribe virus; *Melia azedarach* L.; arenavirus; viral encephalitis.

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We have previously reported[4,5] that crude extracts prepared from roots or leaves of *Melia azedarach* L., (M.a.), contained an antiviral factor which inhibited the in vitro replication of several animal viruses, i.e. Herpes simplex type 1 and 2, Sindbis, Vesicular stomatitis, Foot and Mouth disease virus and the arenaviruses Junín, Tacaribe and Pichinde. Furthermore, pretreatment of different host cells with M.a. extract followed by washing rendered the cells refractory to virus replication.

At present, viral encephalitis cannot yet be controlled by currently available antivirals; drugs which survived in vitro screening programs failed when efficacy was assessed in an intact host, owing to high toxicity and/or lack of the solubility necessary for therapeutical levels to be reached.

The lack of toxicity for mammalian cell cultures shown by M.a. extracts, even at anhibitory concentrations, encouraged us to test the in vivo effect of AVF using an animal model. To evaluate the therapeutic potentialities of M.a. extract against viral encephalitis, we have selected Tacaribe virus (TACV) which induces a lethal encephalitis in new-born mice after i.c. or i.p. infection[6]. The advantage of the TACV model resides in its lack of pathogenicity for man, its close antigenic relationship with Junin virus (agent of Argentine hemorrhagic fever)[7,8] and our long experience in handling arenaviruses.

The present work reports the protective effect of a partially purified AVF obtained from leaves of M.a. on morbidity and mortality in TACV-infected new-born mice, and preliminary studies of its mechanism of action on TACV replication in Vero cells.

Material and methods. Virus: Stocks of TACV, strain 11573, were prepared as 10% infected suckling mouse brain homogenate as described elsewhere[6]. Virus stocks used had a titer of 1.0×10^8 PFU/ml, determined in Vero cells.

Cell cultures: Monolayers of monkey kidney Vero cells were serially propagated in Eagle minimum essential medium (MEM) supplemented with 5% heat-inactivated calf serum and 50 µg/ml gentamycin. Eagle basal medium (BME) with 3% calf serum and antibiotics was used as maintenance medium.

Plant material and partial purification of AVF: M.a. leaves were collected in early spring in the immediate area of Buenos Aires city. Species taxonomy was confirmed at the Department of Botany, Science School, Buenos Aires University, where a herbarium specimen has been kept and registered (Argentina, BAFC 1432). Crude preparations were obtained by homogenization of fresh green leaves in 0.01 M potassium phosphate buffer (KP buffer), pH 7 containing 0.35 M KCl (1 g/ml) at 4 °C in a Waring-type blender, followed by filtration through cheese cloth. All subsequent operations were carried out at 2–4 °C. The dark green extract obtained was centrifuged at 10,000 × g for 1 h and the supernatant dialyzed against KP buffer overnight. The solution was centrifuged to remove turbid material and the supernatant fluid was concentrated 10 times by lyophilization. The powder obtained was resuspended in KP buffer and passed through a column of Sephadex G-100 (Pharmacia, Sweden). The active fractions were pooled and stored at − 20 °C.

Estimation of protein content of AVF using the Lowry reaction was 0.1 mg/ml. The inhibitory activity of AVF was estimated by a plaque reduction method in Vero cells, using TACV. Throughout this paper 1 antiviral unit/ml of AVF is defined as the inverse of the dilution that reduced plaque formation of TACV in Vero cells by 50% in comparison with untreated infected cultures and was calculated from a graphic of the data (% of virus control versus $\log_{10}$ of AVF dilutions). Standard preparations of AVF contained 50 antiviral units/ml.

Mouse infection and AVF dosification: Four-day-old albino swiss mice (strain OF_1 Lyon Credo) were inoculated i.p. with 0.02 ml of a dilution of AVF in PBS equivalent to 0.2 ml of antiviral units per dose. Treatment was initiated 24 h before infection and continued once a day for 6 consecutive days. Control animals received a placebo of PBS following the same schedule.

Effect of AVF on TACV replication in cell culture[a]

Time of AVF addition (h)	Reduction in virus titer ($\log_{10}$ PFU/ml relative to control)
0[b]	3.1
+1	3.3
+3	3.3
+5	2.3
+7	1.4
+10	0.3

[a]Monolayers of Vero cells were infected with TACV at a multiplicity; [b]Virus was inoculated at zero time and was removed after 1 h absorption. AVF was added at indicated times and remained up to 30 h p.i., when supernatants were harvested and titered. Infected cultures without AVF served as controls.

In another experimental design two groups of nine new-born mice were inoculated i.p. with 10^3 PFU of TACV and these animals did not receive direct AVF treatment. Instead, AVF was administered only to their fostering mothers. Treatment consisted of 15 antiviral units in 1.5 ml of PBS given by the i.p. route, starting 24 h before their offspring were infected with TACV and continued for 6 days thereafter. In control groups, neonates were similarly infected but their nursing mothers received PBS following an identical schedule treatment. In both types of experiments, morbidity and mortality were scored daily for 25 days after infection.

The doses administered to neonatal and adult mice resulted in no toxic symptoms; no loss of weight, alterations in behavior or death were observed during the treatment and for a period of 30 days thereafter.

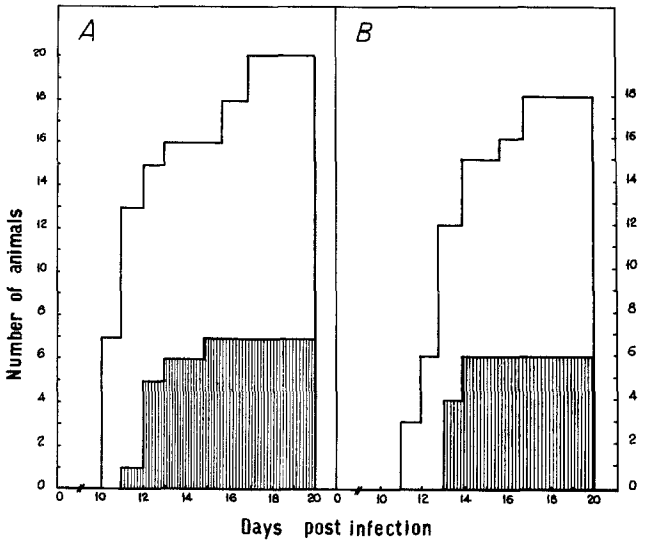
Results. In vitro testing: The inhibitory effect of AVF on TACV replication was studied first by adding AVF after virus adsorption; it remained up to the time of supernatant harvest. The degree of viral yield reduction depended on AVF concentration. The maximum inhibition (3 logs infectivity) was found with a preparation of AVF containing 10 µg/ml of protein, while the lowest active AVF concentration (2.5 µg/ml) reduced infectivity by 1 log unit.

To determine whether AVF could directly inactivate the virus, 1×10^5 PFU of TACV was diluted 10-fold in tubes containing AVF at the maximum inhibitory concentration. Control samples contained the same amount of virus diluted in PBS alone. All tubes were incubated at 37 °C for 2 h. After that time aliquots were removed, diluted ten-fold and titrated in Vero cells. The results obtained showed that there was not a viricidal effect of AVF on TACV, because virus titers were 3.4×10^4 PFU/ml for AVF-exposed virus and 5.6×10^4 PFU/ml for virus incubated in PBS.

We reported previously that pretreatment of host cells with AVF rendered them refractory to virus replication[4]. Taking advantage of this particular property of AVF, its effect on virus attachment to the cell surface was determined by estimating the residual TACV remaining after adsorption on AVF-pretreated and non-treated cultures. Since non-adsorbed virus titers obtained were similar, 1.8×10^3 PFU/ml and 1.7×10^3 PFU/ml for treated and control cultures respectively, it appeared unlikely that the antiviral effect of AVF could be accounted for inhibition of viral adsorption.

In order to determine the step in the viral replication cycle at which AVF exerted its maximum effect, AVF was added to cell monolayers at various times from 2 h before infection to 10 h post-infection and remained present until virus harvest. Supernatants were collected at 30 h p.i. and viral yields determined. The results quoted in the table show that the greatest inhibitory effect was noted when AVF was present during the first 3 to 4 h after adsorption. Addition of AVF after this time interval has a less apparent effect on viral replication.

In vivo testing: Figure A shows the results of direct treatment with AVF of new-born mice infected with TACV at a dose able



A The effect on neonate mortality of AVF given i.p. 24 h before infection and continued once a day for six consecutive days. Animals were infected with 10^4 PFU of TACV (group size 20). *B* The effect of AVF administration to fostering mothers on mortality of neonates infected with 10^3 PFU of TACV (group size 18). ▨, treated; □, untreated.

to kill 100% of inoculated animals. Signs of neurological involvement and paralysis with final evolution to death started at 7 days after virus inoculation in animals that had not received AVF. In this group 20/20 animals died between days 10 and 18 p.i. In the group treated with 8 daily doses of AVF a clear delay of initiation of the disease was noted and only 7/20 animals died between days 11 and 15 after viral inoculation. None of the surviving 13 animals displayed signs of disease. This rate of protection had a statistical signification ($p = 0.005$), according to the Kolmogorov-Smirnov analysis. However, AVF treatment did not alter the mean survival time of the animals that died, since the calculated values were 12.05 and 12.43 days for control and treated groups respectively. When the same experimental design was developed using an inoculum of TACV that killed 50% of inoculated new-born mice, a 100% protection of treated animals was obtained. Using this virus dose, and reducing AVF treatment to 5 daily doses instead of 7, a 100% protection was also obtained.

We want to comment on the results obtained for new-born mice when nursing mothers (not inoculated with virus) were treated with AVF. Littermates were inoculated with TACV at a dose that killed 100% of the animals, but did not receive AVF treatment. The degree of protection against mortality induced by TACV in new-born mice which were fostered by AVF-treated mothers is seen in figure B. A high degree of statistical significance indicated that AVF efficiently prevented development of disease and death in animals that fed from mothers treated with partially purified extract. Variation of TACV dose, particularly using a virus titer that killed 50% of inoculated animals, showed a more dramatic result, because there was no death among the animals that fed from treated mothers (data not shown), and 50% died in the group that did not receive AVF.

Discussion. According to the results reported here the efficacy of systematic administration of AVF against lethal TACV infection in mice is demonstrated. Thus, the effectiveness of AVF against TACV shown in cell-culture can be extended to in vivo infection.

Although the mechanism of antiviral action of AVF against TACV has not been defined yet it was not directly viricidal nor did it affect virus adsorption. Time-of-addition studies indicated that AVF inhibited an early step in the replication process. At concentrations of AVF showing a clear in vitro activity and an in vivo protective effect against TACV there was no indication of toxic effects on the animals such as loss of weight, behavior alterations or death, either in adults or in new-born mice. If we compare it with classical antiviral drugs, AVF shows a promising relationship between protective effect versus toxicity. Experiments to examine effects on fertility and pregnancy are in progress. The inoculation of neonatal mice with TACV induces a dose-response disease, evident from 8–9 days post-inoculation and characterized by ruffled fur, lateralization of locomotion, generalized tremor with progression to ataxia and paralysis of the hind legs either spontaneous or induced by spinning the mice by their tails[6]. The pathogenesis for new-born mice induced by Junin and Tacaribe viruses is probably due to effects resulting from the activation of cellular immune processes against the virus[8,9]. We do not know yet whether AVF inhibits the replication of TACV in vivo, thus we cannot distinguish whether the therapeutic effect of AVF was due to an absolute inhibition of virus replication or occurred because AVF could modulate the cellular immune response. The protection of new-born mice by the treatment of the mother was similar to that obtained by systematic administration of AVF to neonates. These results are difficult to explain. We know that AVF induced in cells a state refractory to virus infection but we were not able to show that a cellular product with similar antiviral activity was excreted into the culture media or transfered to other cells (unpublished data). Alternatively, the protection of weanling mice could be simply explained by the fact that a still-therapeutic concentration of the compound was excreted in the milk of nursing mothers.

The absolute lack of demonstrable toxicity by gross criteria, and the significant protection against lethal encephalitis in mice, demonstrated here, suggest that AVF obtained from *M. azederach* L. may prove useful in the treatment of clinical disease produced by other arenaviruses, especially for neurological forms of Junin virus disease in man[9].

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