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SUPPRESSION OF PERSISTENT VARICELLA-ZOSTER VIRUS INFECTION IN VERO CELLS BY ACYCLOVIR

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Summary. — Persistent infection was established in Vero cells inoculated with varicella-zoster virus (VZV)-infected WI-38 cells. Treatment with 9-(2-hydroxy-ethoxymethyl)guanine (acyclovir) at doses of 100, 80, 40, and 10 μg/ml eliminated the infectious virus, lower doses such as 0.1 and 0.01 μg/ml were ineffective.

Key words: varicella-zoster virus; persistence; acyclovir

VZV is capable of remaining in a latent state in humans after primary infections, and can be reactivated at a later stage, even in the presence of specific circulating antibodies (Barstrain *et al.*, 1974; Jancas, 1979). Although this reactivation usually does not produce serious disease, in immunodepressed patients, however, it may give rise to severe, even fatal consequences (Schimpf *et al.*, 1972; Levin and Zaia, 1977). In this paper we report of persistently VZV-infected Vero cell cultures "cured" by acyclovir.

The WI-38 cells (human embryo lung cells), Flow 7000 (human embryo foreskin cells) and Vero cells (African monkey kidney cells) were purchased from Flow Laboratories Inc. (Cultek), Madrid, and tested for the absence of mycoplasms. The cells were grown in medium Eagle's MEM with Hanks' balanced salts, 0.85 g/l $NaHCO_3$, supplemented with 10% foetal calf serum, 1% glutamine, 1% 100× amino acids solution, 100 U/ml penicillin and 100 μg/ml streptomycin. The maintenance medium had the same composition, except for foetal serum, which was reduced to 5%. VZV isolated from a patient in the second passage in primary cells of human embryonic lung was obtained from the National Centre of Microbiology, Virology and Immunology in Majasahonda, Madrid. VZV was maintained by means of successive passage of infected cell suspensions over monolayer WI-38 cells. The acyclovir was supplied by Burroughs Wellcome Co., Triangle Park, N. C., U.S.A.

The WI-38 cell culture and Flow 7 000 cell culture have proved to be sensitive to VZV multiplication, as they produced infectious centres, visible under the microscope, approximately from the 4th postinfection day. A culture of VZV infected WI-38 cells, showing 50% cytopathic effects (CPE) was trypsinized and inoculated onto monolayer Vero cells. After incubation for 90 min at 37 °C the cell monolayer was washed twice with phosphate-

Table 1. Effect of acyclovir on persistent

Acyclovir (μg/ml)	Days after onset and sampling	Cell confluency (%)	Virus titre (PFU/ml)	Per cent of infected cells
100	1	100	5.00×10^0	0.0025
	4	100	0	0
	→ 7	100	0	0
	11	100	0	0
	14	100	0	0
	18	100	0	0
80	1	100	8.33×10^0	0.0042
	4	100	0	0
	→ 7	100	0	0
	11	100	0	0
	14	100	0	0
	18	100	0	0
40	1	100	1.16×10^3	0.5800
	4	100	5.00×10^1	0.0250
	7	100	0	0
	→11	100	0	0
	14	100	0	0
	18	100	0	0
10	1	100	4.50×10^3	2.2500
	4	100	5.00×10^2	0.2500
	7	100	8.30×10^0	0.0041
	11	100	0	0
	→14	100	0	0
	18	100	0	0

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-buffered saline to remove the inoculum of infected cells and then growth medium was added. CPE was observed since day 2 postinfection, alternative cycles of partial culture destruction and cell recovery developing in subsequent days. During the recovery stage the cells were propagated to obtain persistently VZV-infected Vero cells. Antiviral therapy trials were conducted with acyclovir as described below.

Considering the special features of the VZV culture (cell-associated infection) and the aim pursued, the PICC test (persistent infection cell culture test) as proposed by Schwöbel and Streissle (1976) was applied. This test was programmed according to a pre-established scheme. In our case it consisted of trypsinization of persistently infected Vero cells, and their transfer to flat bottom Nunc tubes, in such a way that after three days a confluent monolayer was observed. The culture medium was then removed and fresh medium with the desired acyclovir concentrations was added. After further incubation (24 hr) at 37 °C, the trypsinized cell samples were titrated for

VZV replication in Vero cells (PICC test)

Acyclovir (μg/ml)	Days after onset and sampling	Cell confluency (%)	Virus titre (PFU/ml)	Per cent of infected cells
1	1	100	6.83×10^3	3.4150
	4	90	1.01×10^4	5.6100
	7	75	1.16×10^2	0.0773
	11	50	2.30×10^1	0.0230
	14	100	1.83×10^3	0.9150
	18	100	3.33×10^1	0.0166
0.1	1	100	7.83×10^3	3.9150
	4	75	2.00×10^4	13.3000
	7	25	5.00×10^2	1.0000
	11	35	1.00×10^3	1.4280
	14	75	4.16×10^3	2.7700
	18	70	8.33×10^2	0.5950
0.01	1	100	8.50×10^3	4.2500
	4	70	3.60×10^4	25.7100
	7	25	2.16×10^3	4.3200
	11	1	5.30×10^1	2.6500
	14	50	1.33×10^3	1.3300
	18	65	3.33×10^2	0.2560
VZV without drug	1	100	1.50×10^4	7.5000
	4	70	1.50×10^4	10.7100
	7	25	1.50×10^3	3.0000
	11	1	1.00×10^2	5.0000
	14	75	4.83×10^3	3.2200
	18	70	5.00×10^2	0.3570

→ The drug was removed and maintenance medium without drug was added.

their virus content of virus-free Flow 7000 cells by infectious centre counts. The titration was carried out in 24 flat bottom well plates (four per sample) on Flow 7000 cell monolayers inoculated with 0.2 ml/sample per well in decreasing concentrations. After 90 min adsorption, the maintenance medium was added and incubated at 37 °C, 95% humidity and 5% CO_2. Four days later, the preparation was stained with crystal violet (2% in ethanol) and the infectious centres were counted under stereomicroscope. Simultaneously, the infected cultures were trypsinized at a split ratio of 1 : 2 and transferred into a new lot of Nunc tubes, to which growth medium with the respective acyclovir concentrations was added. In parallel with the antiviral assays, the compound was examined for its effect on the morphology of normal, uninfected cell cultures, which had been in contact with the compound for the same time period as the virus-infected cells.

As shown in Table 1, subsequent passages and virus titrations were performed to determine the condition of the cell monolayer (confluence and

Table 2. Effect of acyclovir on VZV infected Vero cells after drug pretreatment

Virus titre (infectious centers/ml)	Acyclovir concentration (μg/ml)			
	0	10	40	100
Pretreated cultures	1.63×10^4	2.00×10^3	1.00×10^3	6.60×10^0
No pretreatment*	1.66×10^4	1.83×10^3	1.16×10^3	5.00×10^0

* Pretreatment for 12 days with acyclovir (0.1 μg/ml) continued with given dosis.

cytotoxicity). When virus titres approached to zero, the medium was replenished with fresh growth medium containing no acyclovir, the subculture and titration cycles being continued to check possible reappearance of VZV.

It must be stressed that the PICC test was designed and applied by Schwöbel and Streissle (1976) for persistently infected cell cultures with virus release into the culture medium. In the case of VZV, characterized by its considerable thermal lability, no free infectious virus is found in the culture medium, hence there was a need to titrate the cell-associated virus. On the other hand, fluctuations in virus titre could be observed in drug-free controls of persistently VZV-infected Vero cells. For this reason the percentage of cells forming infectious centres was established and correlated with the results of total cell counts.

The results obtained suggested a straightforward relationship between the acyclovir dose and the period of virus suppression until it disappeared from the cultures. The non cytotoxic doses of 100, 80, 40 and 10 μg/ml proved to be effective, while lower doses, such as 0.1 and 0.01 μg/ml had no antiviral effect. Furthermore it has been proved that virus suppression was definitive, as cured subcultures did not show any signs of reactivation of the infection in an acyclovir-free medium. Continuous surveillance of the potential onset of drug-induced cytotoxic effects allows an efficient monitoring of the specific drug action.

With the purpose of determining a possible induction of resistant mutants, persistently VZV-infected Vero cell cultures were treated with the maximum acyclovir concentration that still permits virus multiplication (0.1 μg/ml), and the culture medium with drug was replaced daily over a period of twelve days. Simultaneously, persistently infected untreated cultures were maintained, according to Streissle's method (Streissle, 1981). When both acyclovir pretreated and nonpretreated cell cultures were exposed for 24 hr to acylovir (doses of 100, 40, and 10 μg/ml), no significant difference in infectious centres formation as determined by virus titration on Flow 7000 cells was found (Table 2) indicating that no acyclovir-resistant mutants were induced under our experimental conditions.

We may conclude that using an application of the Schwöbel/Streissle PICC test, the clear antiviral effect of acyclovir on persistent VZV infection in Vero cells has been demonstrated.

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