INHIBITION OF SEMLIKI FOREST AND HERPES SIMPLEX VIRUS PRODUCTION IN α-DIFLUOROMETHYLORNITHINE-TREATED CELLS: REVERSAL BY POLYAMINES

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1. Introduction

The suggested importance of the natural polyamines putrescine, spermidine and spermine in the macromolecular synthesis and proliferation of animal cells [1,2] has generally been confirmed by results obtained with inhibitors of polyamine synthesis. However, studies of virus production in the inhibitor-treated cells which could serve as a model system to explore the cellular function(s) of polyamines, have revealed conflicting results. A decrease of the formation of vaccinia virus and human cytomegalovirus (HCMV) has been observed [3–5] in cells treated with methylglyoxal bis(guanylhydrazone), an inhibitor of *S*-adenosylmethionine decarboxylase (EC 4.1.1.50), whereas this drug did not affect [4,6] the production of herpes simplex viruses (HSV-1 and HSV-2). Similarly, D,L-α-methylornithine, a competitive inhibitor of ornithine decarboxylase (EC 4.1.1.17), had no effect on the replication of HSV-1 and HSV-2, but decreased the production of HCMV [4]. However, in [7] no effect on the HCMV synthesis was observed in cells treated with α-methylornithine or its derivative, D,L-α-difluoromethylornithine (DFMO). Further studies of this system seemed therefore desirable, with special attention being paid to the effect of the experimental set-up and the specificity of the action of the inhibitor.

Here, we show that the production of two different types of viruses, HSV-2 and Semliki Forest virus, is inhibited in cells pretreated with DMFO. This inhibition could be abolished by exogenous polyamines.

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2. Materials and methods

BHK21, a continuous cell line derived from baby hamster kidney, and Vero cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum, penicillin (200 U/ml) and streptomycin (100 μg/ml). A prototype strain of Semliki Forest virus (SFV) was obtained from Dr L. Kääriäinen (University of Helsinki) and strain 333 of herpes simplex virus type 2 (HSV-2) from Dr F. Rapp (Pennsylvania State University, Hershey). Stock cultures of both viruses were grown in BHK21 cells, and infectivity, as plaque forming units (p.f.u.), was determined in BHK21 cells for SFV and in Vero cells for HSV-2.

D,L-α-Difluoromethylornithine was a generous gift from Centre de Recherche Merrell International, Strasbourg. Polyamines obtained from commercial sources were purified as in [8]. Iproniazid phosphate was a product of Hoffmann-La Roche.

The experiments were started by subculturing BHK21 cells in plastic Petri dishes (diam. 50 mM) at a ratio of ~1:10 (3.5×10^5 cells/dish). The cells were grown in the presence of 1 mM iproniazid (an inhibitor of serum amine oxidase, see [9]) and in the presence or absence of DFMO and 10 μM polyamine before infection with SFV (50 p.f.u./cell) or with HSV-2 (5 p.f.u./cell) as in [9,10]. After adsorption for 1 h the maintenance medium (MEM containing 0.2% bovine serum albumin and the same additions as the corresponding growth medium) was added. The production of SFV was determined from samples of the medium 8 h post infection and that of HSV-2 from cell sonicates prepared in the medium at 20 h post infection. Virus titers were expressed as p.f.u./μg

cellular DNA. DNA was determined according to [11] from similarly treated but non-infected cultures.

3. Results and discussion

D,L-α-Difluoromethylornithine has been shown to effectively decrease the concentrations of putrescine and spermidine in cultured rat hepatoma and mouse leukemia cells, and also in tumor cells in vivo, followed by inhibition of cell replication [12,13]. In agreement with these observations, treatment of BHK21 cells for 24 h with 2 mM DFMO markedly diminished cellular putrescine and spermidine, whereas the concentration of spermine did not change or was even increased (not shown). There was little influence on the accumulation of DNA and RNA during the first 24 h, but after 2-day treatment the net accumulation of nucleic acids was almost completely prevented. A similar delay was observed in the inhibition of protein synthesis as measured by the incorporation of [^{14}C]leucine. The inhibition of macromolecular synthesis and cell proliferation by DFMO was reversed by 10 μM spermidine added to the growth medium.

The delay in the appearance of the inhibition of virus production in DFMO-treated cells was even longer than that observed in cellular nucleic acid synthesis. As seen in table 1, the yield of HSV-2 and SFV was even increased during the first 2 days, but was clearly decreased after treatment for 72 h. In later experiments a pretreatment of 72 h was used.

As is seen in table 2, 10 μM spermidine added at various times during the pretreatment with DFMO abolished the inhibition of virus production. In the case of HSV-2 the reversal appeared fairly slowly, but was complete in 24 h in the case of SFV. As shown in table 3, the addition of 10 μM spermidine at 12 h before infection partly reversed the inhibition.

Next the specificity of the reversal by polyamines was studied. As may be seen from table 4, putrescine and spermidine added 24 h before infection completely reversed the inhibition of SFV production. Cadaverine was somewhat less effective and no effect was found with 1,3-diaminopropane or spermine. Because the latter may only slowly penetrate the cell membrane, additional studies were carried out with sperm-

Table 1
HSV-2 and SFV production in BHK21 cells pretreated with 5 mM DFMO

Time of pretreatment (h)	Virus production (%)	
	HSV-2	SFV
0[a]	120	185
24	137	192
48	128	123
72	42	33

[a] DFMO added after virus adsorption

The number of viruses in control cultures (2.73×10^3 p.f.u. for HSV-2 and 1.3×10^7 p.f.u./μg cellular DNA for SFV) was taken as 100%. All values are means of 2 parallel dishes

Table 2
Effect of spermidine on HSV-2 and SFV production in DFMO-treated cells

Time of spermidine addition before infection (h)	Virus production			
	HSV-2		SFV	
	Control	DFMO (%)	Control	DFMO (%)
No spermidine	4.1×10^5	19	3.5×10^6	11
24	3.8×10^5	53	4.0×10^6	113
48	5.1×10^5	49	3.9×10^6	149
72	4.7×10^5	96	3.1×10^6	123

BHK21 cells were grown for 72 h in the absence (control) or presence of 2 mM DFMO before infection. Spermidine (10 μM) was added at indicated times to both control and DFMO-treated cultures

Table 3
Reversal by spermidine of the inhibition of SFV production in DFMO-treated cells

Time of spermidine addition before infection (h)	Virus production	
	p.f.u./μg DNA	%
No spermidine	0.78×10^6	4
No spermidine[a]	1.17×10^6	7
2	2.34×10^6	13
4	1.50×10^6	9
8	2.92×10^6	17
12	1.08×10^7	62
24	1.95×10^7	112

[a] Spermidine added after adsorption

BHK21 cells were grown for 72 h in the absence or presence of 2 mM DFMO. Iproniazid (1 mM) was added at 48 h to all cultures. Virus production in the control culture (1.74×10^7 p.f.u./μg DNA) was taken as 100%

Table 4
Effect of various polyamines on SFV production in DFMO-treated cells

Additions	Virus production	
	p.f.u./μg DNA	%
Control	7.0×10^6	100
DFMO	6.3×10^5	9
DFMO + 1,3-diaminopropane	7.4×10^5	11
DFMO + putrescine	1.0×10^7	143
DFMO + cadaverine	5.6×10^6	80
DFMO + spermidine	7.1×10^6	101
DFMO + spermine	4.8×10^5	7

BHK21 cells were grown for 72 h in the presence of 2 mM DFMO. Iproniazid (1 mM) and polyamines (10 μM) were added 24 h before infection

ine. The results (not shown) demonstrated that spermine effectively increased virus yield in DFMO-treated cells when added 48–72 h before infection.

From our results it appears that the effect of inhibitors of polyamine synthesis on virus production is dependent on several factors, e.g., the specificity of the inhibitor, the duration of pretreatment, the cell density at the start of pretreatment. These factors seem to explain at least partly the discrepancy in the earlier results (see section 1). It has been shown that some inhibitors, e.g., 1,3-diaminopropane and methylglyoxal bis(guanylhydrazone), have acute effects unrelated to polyamine deficiency [9,14]. DFMO, being a potent and specific inhibitor of polyamine synthesis and showing a remarkably low toxicity in tissue cultures and whole animals [13], is clearly superior to these drugs. A short pretreatment time possibly explains why α-methylornithine and methylglyoxal bis(guanylhydrazone) did not affect the replication of herpes simplex viruses in some earlier studies [4,6]. In [7] DFMO did not inhibit the production of HCMV. In that study confluent or nearly confluent cell cultures were used, which may delay the appearance of polyamine deficiency.

Our data strongly suggests that polyamines or polyamine-dependent factor(s) are needed for HSV-2 and SFV replication. This view is supported by the observation that the inhibition of virus production by DFMO was reversed by exogenous polyamines. The inhibition of vaccinia virus replication by methylglyoxal bis(guanylhydrazone) was also reversed by spermidine in [3], but in this case the interpretation is complicated by the finding that the inhibitor and the polyamines compete for a common uptake system [15]. The mechanisms by which polyamine deficiency interferes with virus production are not known. Virus replication consists of several steps which may be sensitive to polyamine depletion. As pointed out [16], the sensitive step(s) may be virus specific and/or include a malfunction of cellular macromolecule synthesis. e.g., at the ribosomal level. We are currently investigating the target site(s) of polyamine depletion in the virus replication cycle and in the host macromolecular synthesis.

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