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THE EFFECT OF PRE-INHIBITION OF DNA SYNTHESIS IN CONTINUOUS HUMAN AMNIOTIC CELL CULTURE (STRAIN FL) ON ADENOVIRUS TYPE 5 REPRODUCTION

E. S. ZALMANZON, L. N. MIKHAILOVA

Institute of Molecular Biology, U.S.S.R. Academy of Sciences, Moscow

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Summary. — The effects of pre-inhibition of DNA synthesis in human amniotic cell culture (strain FL) on the reproduction of adenovirus type 5 were studied using inhibitors (aminopterin, 5-fluorouracil, 5-fluorodeoxyuridine) and irradiation with X and ultraviolet (UV) rays. The degree of DNA synthesis inhibition was estimated from ^{3}H-thymidine incorporation. All these agents inhibited adenovirus reproduction. The less intensive virus reproduction in DNA-inhibited cells could not have been due only to a decrease of nucleotide pool in the cell (in contrast to DNA inhibitors, UV irradiation does not affect the nucleotide pool) or to death of some of the cells during treatment. Counting of cells synthesizing the viral antigen showed that there occurred a decrease of the virus titre but also of the percentage of cells synthesizing viral antigen. A possible role of the host genome in the mechanism of adenovirus type 5 reproduction is discussed.

Introduction

In virus-cell interaction studies, it is of interest to explore to what extent the virus is autonomous inside the host cell. It is known that most of the RNA-containing viruses do not require the participation of cell genome for their reproduction, which can take place in the presence of inhibitors of cellular DNA and RNA and even in cells pre-irradiated with UV or X rays that are known to inhibit DNA synthesis (Bader, 1966; Barry *et al.*, 1962; Fenwick, 1963; Kritschevsky *et al.*, 1963; Loh, 1960; Reich and Franklin, 1961; Rosenbergová and Rosenberg, 1962; Rott *et al.*, 1965; Shatkin, 1962). Exclusion of host-cell genome often even reduced the latent phase of infection (Bukrinskaya and Zhdanov, 1963). An exception are influenza and Rous sarcoma viruses which are sensitive to actinomycin D and cannot reproduce in cells pre-irradiated with X or UV rays (Barry *et al.*, 1962; Bader, 1964; Rott *et al.*, 1965; Temin, 1963; White and Cheyne, 1966; Rubin and Temin, 1959). Influenza viruses require the host genome within the first 80—150 minutes post infection when viral particles are being incapsidated (White and Cheyne, 1966). In contrast, Rous sarcoma virus not only requires the host genome, but also stimulates the synthesis of its DNA (Temin, 1964*a*, *b*).

The dependence of DNA-containing virus reproduction on the function and synthesis of cellular DNA has not yet been sufficiently studied, and the available results are often contradictory. Thus, for instance, Bowen *et al.* (1966) have shown that pre-irradiation of mouse embryo fibroblasts with UV makes them unable to sustain polyoma virus reproduction, while Habel (1967) reported an increased production of T-antigen and infectious polyoma virus in the same cultures pre-irradiated with non-lethal UV doses. According to Wassermann (1965), UV irradiation of HeLa cell culture inhibits cell reproduction, both prior to and after infection with adenovirus types 1 or 4. On the other hand, Harnois *et al.* (1966) described DNA synthesis activation in Syrian hamster and human embryo cell cultures infected with adenovirus types 5 and 12 following X-irradiation in doses inhibiting DNA synthesis. Harnosis *et al.* claimed to have observed the formation of a complete virus and its antigen in irradiated human embryo cell cultures. Host genome participation in liberating vaccinia virus from the protein coat was pointed out by Joklik (1964).

In the present study we investigated adenovirus type 5 reproduction in cells with inhibited DNA synthesis and also the dependence of reproduction of this virus on the mode of DNA synthesis inhibition in host cells.

Materials and Methods

Cell cultures. A continuous human amnion cell culture (strain FL), grown as described previously (Zalmanzon and Liapunova, 1963), was used. For experiments on inhibitors and X-irradiation, the cells were grown in monolayers on tube walls, and for experiments on UV-irradiation on 0.8×7.0 cm glass slides placed in tubes containing 5.0 ml of medium.

Virus. Adenovirus type 5 was obtained from the Ivanovsky Institute of Virology, U.S.S.R. Academy of Medical Sciences; it was maintained by passaging in FL cell cultures. The virus-containing culture fluid was collected after degeneration of infected cells, centrifuged for 30 minutes at 2,500 rev/min and stored at $-15°$ C.

DNA-inhibitors. 5-Fluorouracil, syntesized by B. A. Igin, was used in the concentration of 25 μg/ml; 5-fluorodeoxyuridine and aminopterin were used in concentrations of 0.01 and 45 μ/ml, respectively. The preparations were diluted in Eagle's medium containing 2% calf serum and added to a 24-hour-old cell culture 20 hours before infection.

Labelled gamma-globulin was derived from hyperimmune rabbit sera by the method described previously (Zalmanzon and Liapunova, 1963), conjugated with fluorescein isothiocyanate, purified according to the method of Mekler and Naumova (1965), and stored at $-15°$ C.

X-irradiation. Twenty hours old monolayer cell cultures on tube walls or on glass slides in tubes, after replacement of the nutrient medium by Hanks' solution, were irradiated by an X-ray installation (type RUP-200-20), with the tube voltage of 170 kv and anode current of 15 ma. The focal distance was 25 cm and this provided for a dose of 325 rad/min. After irradiation, Hanks' solution was replaced by fresh Eagle's medium with 2% calf serum. Some of the tubes were used for virus infection and some to measure DNA synthesis.

UV-irradiation. Slides with 48 hours old cultures were placed into Petri dishes containing 12 ml of 0.85% NaCl solution and irradiated with doses of 150 and 250 ergs/mm^2 while in open dishes at a distance of 74 cm from a bactericidal UV lamp (type BUV-15). The slides were then placed into tubes with nutrient medium or virus.

DNA synthesis was estimated from ^{3}H-thymidine incorporation, for which purpose the slides were placed into Petri dishes with saline containing 0.5 μc/ml of ^{3}H-thymidine and incubated for 30 minutes at 36° C. After washing in cold saline, the cells were fixed by 70% ethanol and washed in 2% perchloric acid and water; to prepare autoradiograms, the cells

were covered with liquid emulsions M and P, manufactured at the Scientific Research Institute of Cinematography, according to the usual procedure (Zelenin and Liapunova, 1961). The rate of label incorporation was estimated from the number of silver grains on the nuclear surface. Grains were counted in 300 cells on three preparations. The results obtained were subjected to statistical treatment.

In some of the experiments, DNA synthesis was assessed on the basis of ^{3}H-thymidine incorporation into the cell fraction insoluble in trichloroacetic acid. In this case, after incubation with ^{3}H-thymidine, cells were dispersed by ethylenediamine tetraacetate (EDTA), collected on Millipore filters RUFS, and washed on the filters 4 times with 0.85% NaCl solution, 5 times with 5% trichloroacetic acid and 2 times with ethanol, after which radioactivity was measured in a scincillation counter. Average results were obtained by collecting cells from 3 slides on a single filter and by using 6 slides in each experiment.

Virus reproduction. Cells were infected with adenovirus immediately after X- or UV-irradiation or after 20 hours of incubation with inhibitors. In the latter case they were thrice washed free from the inhibitor by 5 ml of Hanks' solution. After 1 or 2 hours of adsorption at 36° C, the cells were thrice washed free from non-adsorbed virus and then incubated at 36° C in Eagle's medium containing 2% calf serum. Cells were removed by adding EDTA immediately and 24, 28, 32 and 48 hours after virus adsorption, washed with Hanks' solution and suspended in Eagle's medium without serum. To obtain mean results, cells were collected from 6 test tubes. After fourfold freezing at −60° C, thawing at 35° C and centrifuging, the titre of intracellular virus was measured according to its cytopathic activity in FL cell cultures. The material of each experiment was twice titrated and mean titres were calculated.

The cells synthesizing viral antigen were counted by the method of immunofluorescence described previously (Zalmanzon and Liapunova, 1963).

Results

In the first series of experiments, we studied adenovirus reproduction in cells pretreated for 20 hours with various DNA inhibitors. These included

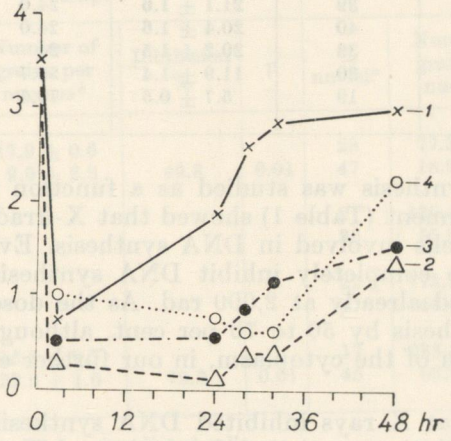

Fig. 1.

Effect of pre-incubation of FL cells with DNA inhibitors on adenovirus type 5 reproduction
Abscisa: virus titre in log $TCID_{50}$/0.1 ml values; ordinate: hours after virus adsorption

1 — Eagle's medium
2 — Aminopterin followed by Eagle's medium
3 — 5-Fluorouracil followed by Eagle's medium
4 — 5-Fluorodeoxyuridine followed by Eagle's medium

5-fluorodeoxyuridine, which is a specific inhibitor of thymidilate synthetase, and also 5-fluorouracil and ametopterin which are inhibitors of thymidine synthesis (Hartmann and Heidelberger, 1961; Eidinoff *et al.*, 1959).

The results obtained are presented in Fig. 1. It can be seen that cells treated with, and washed free of inhibitors lost their ability to sustain adenovirus reproduction. The pre-treatment apparently had no effect on the ability of cells to adsorb the virus, since cell extracts possessed some infectivity. However, increase in virus titre was small, if any. Such an inhibition of virus reproduction could have been due either to (1) a decrease in the nucleotide pool in the cells; (2) inadequate liberation of the cells from inhibitors which still continued to directly affect the viral DNA synthesis; and (3) the virus requirement for continued DNA synthesis in the cell.

To test these assumptions, DNA synthesis in cells was inhibited by X- and UV-irradiation which do not induce the breakdown of nucleic acids or a change in the nucleotide pool in the cells, although their mechanisms of action are different (Powell, 1962; Whitmore *et al.*, 1958).

Table 1. Action of X-rays on ^{3}H-thymidine incorporation into FL cells; 48 hours after irradiation

X-ray dose (rad)	Time of irradiation (min)	% of DNA synthesizing nuclei	Number of grains per nucleus	Inhibition per cent	t	Number of mitoses per 1000 cells
0 (control)	0	40	27.6 ± 1.7			22.6
2000	6.5	43	26.2 ± 1.7	0	<3	0
2000	10	39	21.1 ± 1.6	24.0	<3	0
4000	13	40	20.4 ± 1.6	24.0	<3	0
6000	19.5	33	20.2 ± 1.5	24	<3	0
8000	26	30	11.9 ± 1.4	56.7	>3	0
10000	32.5	19	5.7 ± 0.5	79.5	>3	0

Initially, DNA synthesis was studied as a function of X-ray dose. The results of this experiment (Table 1) showed that X-irradiation had no effect on the number of cells involved in DNA synthesis. Even doses as high as 10,000 rad failed to completely inhibit DNA synthesis, although mitoses were fully suppressed already at 2,000 rad. As the dose of 20,000 rad also inhibited DNA synthesis by 50 to 70 per cent, although it induced an appreciable vacuolation of the cytoplasm, in our further experiments we used a dose of 10,000 rad.

Table 2 shows that X rays inhibited DNA synthesis immediately after irradiation, this inhibition persisting for 24 hours. Therefore we were able to study the effect of irradiation on a single virus replication cycle. It is seen from Fig. 2 that DNA inhibition in FL cells was accompanied by a certain inhibition of virus reproduction as well.

Table 3 illustrates the results of our autoradiographic study of DNA synthesis in cells after UV-irradiation. A dose of 150 ergs/mm^2 inhibited DNA

synthesis by an average of 60 per cent in the first 24 hours after irradiation and led, as also reported by Terskikh (1966), to an increased percentage of

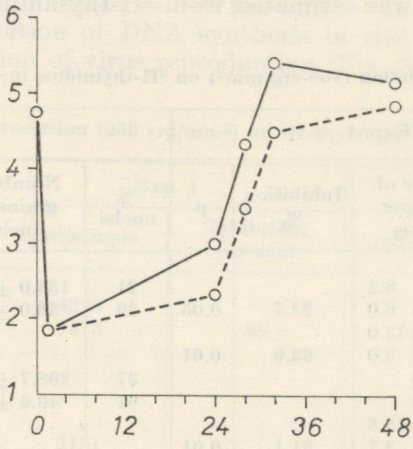

Fig. 2.

Adenovirus type 5 reproduction in cells pre-irradiated with X rays (10,000 rad)
Abscissa and ordinate as in Fig. 1
——— Non-irradiated cells
- - - - - Irradiated cells

Table 2. ^{3}H-thymidine incorporation into FL cells after X-irradiation (10000 rad)

Hours after irradiation		Exp. 1				Exp. 2			
		% nuclei*	Number of grains per nucleus*	Inhibition %	p	% nuclei*	Number of grains per nucleus*	Inhibition %	p
0	C	33	17.9 ± 0.6			28	77.3 ± 15.8		
	I	6	9.0 ± 2.8	49.8	0.01	47	18.9 ± 0.8	75.6	0.01
10	C					27	130.5 ± 14.2		
	I					36	57.8 ± 4.7	55.7	0.01
20	C					22	173.3 ± 16.3		
	I					47	46.5 ± 3.1	73.2	0.01
24	C	34	115.3 ± 9.7			17	235.3 ± 36.4		
	I	56	23.9 ± 1.6	79.3	0.01	45	88.9 ± 6.5	62.3	0.01

C = Unirradiated controls; I = irradiated cells.
* DNA-synthesizing nuclei.

^{3}H-thymidine-incorporating cells and a decreased rate of DNA synthesis in each cell. Fig. 3-I shows that adenovirus reproduction in irradiated cells

was appreciably inhibited. As increased UV doses can induce a more pronounced inhibition of DNA synthesis, we studied DNA synthesis and virus reproduction in cells exposed to a dose of 250 ergs/mm². In these experiments, DNA synthesis was estimated from ^{3}H-thymidine incorporation into

Table 3. Effect of UV-irradiation (150 erg/mm²) on ^{3}H-thymidine incorporation into FL cells

Hours after irradiation		Exp. 1				Exp. 2			
		% nuclei	Number of grains per nucleus	Inhibition %	p	% nuclei	Number of grains per nucleus	Inhibition %	p
0	C	33	81.1 ± 8.4			21	133.0 ± 19.3		
	I	50	62.7 ± 6.0	22.7	0.05	59	36.0 ± 3.0	72.9	0.01
8	C	46	152.8 ± 12.0						
	I	61	55.2 ± 5.0	63.9	0.01				
16	C					37	208.7 ± 14.4		
	I					61	40.6 ± 2.1	80.6	0.01
21	C	28	200.9 ± 18.8						
	I	64	38.0 ± 3.7	81.1	0.01				
22	C	29	188.0 ± 23.1			26	141.0 ± 14.9		
	I	79	84.2 ± 4.2	55.2	0.01	84	53.6 ± 2.2	62.0	0.01
24	C	26	208.4 ± 22.5			27	92.8 ± 11.2		
	I	69	78.0 ± 4.2	62.6	0.01	79	49.0 ± 2.0	47.2	0.01

Only DNA-synthesizing nuclei were taken into account.

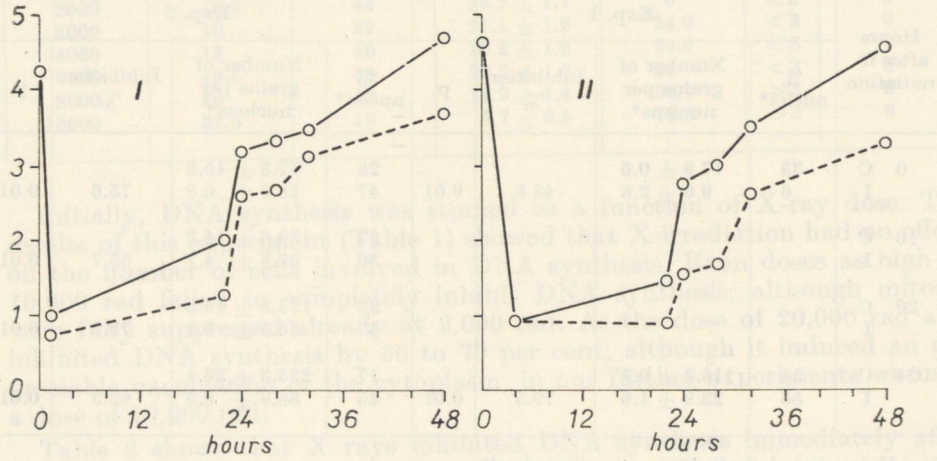

Fig. 3.

Adenovirus type 5 reproduction in cells pre-irradiated with UV rays (I — 150 ergs/mm²; II — 250 ergs/mm²)

Explanations as in Fig. 2.

the trichloroacetic acid-insoluble cell fraction. Table 4 shows that DNA synthesis was inhibited by 40 to 65 per cent immediately after irradiation, with a further inhibition up to 74—89 per cent 8—16 hours after irradiation. The increasing inhibition of DNA synthesis in the cells was accompanied by enhanced inhibition of virus reproduction (Fig. 3-II).

Table 4. Effect of UV-irradiation (250 erg/mm²) on ^{3}H-thymidine incorporation into FL cells

Hours after irradiation	Cells	Exp. 1		Exp. 2	
		cpm/sample	Inhibition per cent	cpm/sample	Inhibition per cent
0	C	4224		4871	
	I	1515	65	3014	38.2
2	C			3218	
	I			1030	53.3
4	C			3609	
	I			1030	71.5
8	C	3151		5520	
	I	342	89	1463	73.5
12	C			5905	
	I			1227	79.3
16	C	7017		7291	
	I	1492	79.0	904	87.6
20	C			5408	
	I			1692	68.7
21	C	6069		5772	
	I	1933	68.0	1435	75.1
22	C			5585	
	I			1467	73.6
23	C	2436		5395	
	I	1228	50.0	1002	81.5
24	C	2400		6464	
	I	1555	35.0	1378	78.7
28	C	4600		6216	
	I	1529	72.0	2830	64.5

C and I: control unirradiated and irradiated, respectively.

Our experiments thus showed that, irrespective of the mode of DNA synthesis inhibition, the cells lost some of their ability to sustain adenovirus reproduction. However, in no case was it possible to exclude with certainty the death of some of the cells. Therefore the question arose as to whether the inhibition of virus reproduction depended on DNA inhibition in the cells or on a decrease in cell number. To answer this question, direct counts of cells synthesizing the viral antigen (per 1,000 cells in the preparation) were made. For this purpose, both UV-irradiated and non-irradiated cells were inoculated with adenovirus, incubated at 36° C for 28 and 46 hours, and after fixation with acetone, stained with specific labelled gamma-globulin according to the procedure described earlier (Zalmanzon and Liapunova,

1963). Cells present on three slides were then counted (at least 500—1,000 cells per slide) in each experiment, and the percentage of fluorescent cells was determined. The results (Table 5) showed that pre-irradiation of cells with UV rays inhibited their ability to support the synthesis of viral antigen.

Table 5. Formation of specific adenovirus type 5 antigen in FL cell cultures after UV-irradiation

UV dose (erg/mm²)	Hours after virus adsorption	Number of fluorescent cells per 1000 cells		
		Exp. 1	Exp. 2	Exp. 3
0 (control)	28		28.2	27.3
	46	19.2	58.1	73.2
150	28		9.5 (66.4%)	13.3 (49.5%)
	46		26.9 (53.7%)	21.7 (70.4%)
250	28		10.8 (61.7%)	4.8 (82.5%)
	46	9.9 (48.5%)	34.3 (41.0%)	9.9 (86.5%)

In parentheses: inhibition per cent.

Discussion

Our experiments demonstrated that, irrespective of the mode of inhibition of DNA synthesis, such inhibition involves a decrease in the cell capacity to support adenovirus type 5 reproduction.

The less intensive virus reproduction in the DNA-inhibited cells could not have been due to a decrease in the nucleotide pool in the cells or to the death of some of the cells as a result of treatment. For, in contrast to inhibitors, UV and X radiation did not affect the nucleotide pool, and direct counting of the percentage of cells that synthesized the viral antigen made it possible to reveal the actual decrease in the number of cells capable of sustaining virus reproduction. It may be concluded, therefore, that adenovirus does rely for its reproduction on host genome participation. Considering that adenovirus, unlike other DNA-containing viruses, do not stimulate DNA-polymerase in the infected cells (Green, 1966), it may be assumed that they utilize that and, possibly, other enzymes of host cells. Moreover, the possibility cannot be excluded that the host genome is involved in the release of viral particles from the protein coat, as it has been shown to take place with some other viruses (Joklik, 1964; White and Cheyne, 1966).

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