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EFFECTS OF INHIBITORS OF DNA SYNTHESIS ON HAEMAGGLUTININ AND INFECTIOUS SV 15 VIRUS FORMATION IN GREEN MONKEY KIDNEY CELLS

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Summary. — The effects of 5-fluoro-2'-deoxyuridine (FUDR) and 5-iodo-2'-deoxyuridine (IUDR) on haemagglutinin synthesis, virus infectivity and the development of cytopathic changes in green monkey kidney (GMK) cells infected with SV 15 virus were studied. Both inhibitors suppressed the synthesis of infectious virus. Haemagglntinin formation was suppressed by FUDR, but not by IUDR. Neither inhibitor suppressed the cytopathic effect (CPE) characteristic of adenoviral infection. Electron microscopy revealed that FUDR completely inhibited adenovirus synthesis but that it apparently did not substantially affect the ultrastructural changes characteristic of adenoviral infection of cells. In the presence of IUDR, the formation of morphologically mature, but noninfectious viral particles was observed.

Introduction

The effects of inhibitors of DNA synthesis on the reproduction of human adenoviruses has been studied by many authors (Green, 1962; Flanagan and Ginsberg, 1962; Kjellen, 1962; Kjellen *et al.*, 1963; Seto *et al.*, 1964; Bauer and Apostolov, 1966; Feldman and Rapp, 1966).

Fong *et al.* (1968*b*) reported about the effect of some inhibitors on the synthesis of infectious SV 15 virus, its complement fixing antigen and the development of the CPE in rhesus monkey kidney cells.

The present communication deals with the effects of FUDR and IUDR on the reproduction of infectious SV 15 virus and its haemagglutinin. Results concerning the synthesis of noninfectious virus and haemagglutinin in the presence of IUDR will also be reported.

Materials and Methods

Cell cultures and media. GMK cells were grown in Hanks' solution with 0.5% lactalbumin hydrolysate and 5% bovine serum. After inoculation with virus, the cell cultures were maintained in medium 199.

Virus. The strain of SV 15 adenovirus, isolated by Gavrilov *et al.* (1963), was used. It was passed in GMK cell cultures and was not contaminated by any haemagglutinating virus. The

virus stock used in the present experiments was prepared in GMK cells and kept frozen at −20° C.

Immune sera to SV 15 virus were produced in rabbits at the Tarasevich State Control Institute.

Inhibitors. FUDR was obtained from the Institute Goustave Roussy, Villejuif, France, and IUDR was a product from the Nutritional Biochemical Corporation (U.S.A.).

Virus titration. The plaque technique was employed. GMK cell cultures in flasks, after thorough removal of growth medium, were inoculated with tenfold dilutions of test material (0.1—0.2 ml per flask). After 90 minutes, adsorption at room temperature, the cell monolayers were overlaid with nutrient agar. The composition of the latter was that described by Altstein and Dodonova (1968). The results were read after 14 days.

Haemagglutination (HA) tests were carried out with 0.4 ml volumes on plastic trays at 4° C. Twofold serial dilutions of virus-containing materials in phosphate buffered saline (PBS) at pH 7.0 were mixed with equal volumes of a 1% suspension of human 0 erythrocytes. The results were scored by the two-cross system.

Haemagglutination inhibition (HI) tests were carried out with 0.6 ml volumes: 0.2 ml of virus-containing material (4 HA units) was mixed with 0.2 ml of twofold dilutions of immune or normal serum previously absorbed with kaolin and human erythrocytes; after 1 hour at room temperature, 0.2 ml of 1% erythrocyte suspension was added.

Electron microscopy. GMK cell monolayers grown in Petri dishes were fixed with 1% glutaraldehyde in medium 199, pH 6.9, for 5 minutes at 4° C and post-fixed in 1% OsO_4 in Sjöstrand's solution, pH 7.2, for 45 minutes at room temperature. The cells were then embedded into a 1 : 4 mixture of methyl and butyl methacrylate by the method of Bykovsky (1961). Thin sections prepared with a Porter-Blum ultramicrotome were stained with uranyl acetate and lead citrate according to Venable and Cogeshall (1965) and examined in a JEM 6-C electron microscope.

Results

One-step growth curve of SV 15 adenovirus

GMK cell cultures in tubes were infected with SV 15 at an input multiplicity of 5 plaque forming units (PFU) per cell. After 2 hours of adsorption at room temperature, the non-adsorbed virus was removed by 5 washings

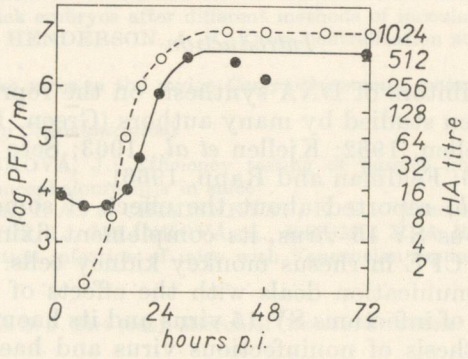

Fig. 1.

One-step growth curve of adenovirus SV 15

● — Infectious virus, ○ — haemagglutinins

with medium 199. Then maintenance medium was added and the cultures were incubated at 37° C. At intervals, two tubes each were removed and stored at —20° C until examined. Before titration, the tubes with cells and maintenance medium were subjected to 2 cycles of freezing and thawing.

A typical result is presented in Fig. 1. The latent period, which elapsed before detectable production of infectious virus and haemagglutinin, lasted 12—16 hours. The exponential reproduction period occurred from 16 to 24 hours after infection (p.i.). Maximal titres of infectious virus and haemagglutinin were observed from 30—48 hours p.i. First signs of a CPE in cell monolayers appeared after 16—18 hours p.i. and the CPE reached its maximum at 48—72 hours p.i.

Table 1. The effects of the concentration of DNA synthesis inhibitors on the reproduction of SV 15 adenovirus in GMK cells

Inhibitor	Inhibitor concentration μg/ml	Inhibition of virus reproduction		
		Exp. 1	Exp. 2	Exp. 3
FUDR	10	ND	+	±
	25	+	+	+
	50	++	++	++
	100	++	++	++
IUDR	10	−	−	−
	25	−	−	−
	50	+	+	+
	100	++	++	++

GMK cell cultures in tubes were simultaneously infected (input multiplicity of 0.01 PFU per cell) and supplied with medium containing the inhibitor; controls without inhibitors were included. The degree of inhibition of adenovirus reproduction was evaluated according to the CPE inhibition:

−, no inhibition;
+, partial inhibition;
++, complete inhibition of the CPE and thus of virus reproduction.
ND = not done.

Estimation of FUDR and IUDR concentrations inhibiting the reproduction of SV 15 adenovirus

Cell cultures in tubes were inoculated with small doses of virus (1000 PFU per tube) and maintained in medium 199 containing different concentrations of FUDR and IUDR. Virus reproduction was estimated according to the CPE. Since the input multiplicity of infection was rather low (0.003 PFU per cell), the appearance of the CPE offered good evidence of virus reproduction and of its spread in the monolayer. The results are presented in Table 1. It is evident that 50 μg/ml of FUDR and 50—100 μg/ml of IUDR were required to obtain complete suppression of SV 15 adenovirus reproduction in GMK cells. In all further experiments reported below, a 100 μg/ml concentration of either inhibitor was employed.

The effects of DNA synthesis inhibitors on the reproduction of infectious virus, haemagglutinin synthesis and the CPE

GMK cell cultures were infected as described above in one-step growth curve experiments. The maintenance medium was supplemented with

FUDR or IUDR in a concentration of 100 μg/ml. Control infected cultures were maintained in medium without inhibitors. Fig. 2 illustrates the results of a typical experiment. The inhibitors completely suppressed the formation of infectious virus. Haemagglutinin synthesis was intensively inhibited by FUDR and only little so by IUDR. HI tests revealed no differences between haemagglutinins produced in control and inhibitor-treated cultures. Neither inhibitor produced an obvious effect on the development of the specific CPE.

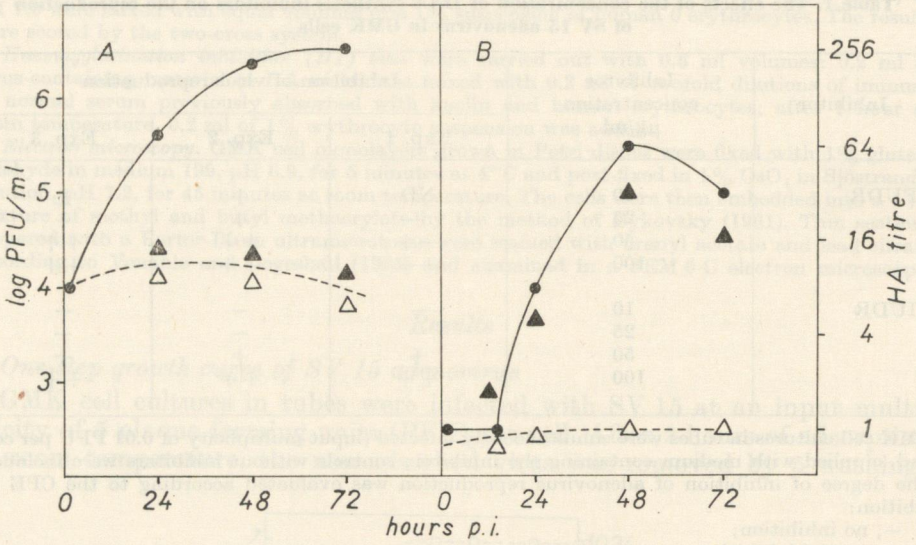

Fig. 2.

Effects of FUDR and IUDR on the reproduction of infectious virus (A) and haemagglutinin synthesis (B) of adenovirus SV 15

▲ IUDR 100 μg/ml
△ FUDR 100 μg/ml
● Control

The effect of the time interval between inoculation (input multiplicity of 5 PFU per cell) and addition of inhibitors on infectious virus synthesis was also studied. The results are presented in Fig. 3. The addition of inhibitors within 24 hours p.i., especially within 12 hours p.i., resulted in marked suppression of infectious virus formation. Moreover, FUDR added within the first 12 hours p.i., inhibited haemagglutinin synthesis; when added later, it failed to do so. IUDR did not affect haemagglutinin synthesis.

Electron microscope study of SV 15-infected GMK cells

GMK cells grown in 6-cm Petri dishes were infected with an input multiplicity of 3 PFU per cell. After 2 hours of adsorption at room temperature, the unadsorbed virus was removed by 5 washings with medium 199. Then maintenance medium without (control) or with 100 μg/ml FUDR or IUDR was added to the cultures. At intervals of 16, 24, 48 and 72 hours p.i. the

cell cultures were fixed for electron microscopy and simultaneously samples were taken for titrations of infectious virus and haemagglutinin.

The course of virus reproduction and haemagglutinin synthesis in these experiments was similar to that shown in Fig. 2.

Control cultures. Electron microscopy of GMK cells fixed at 16 hours p.i. revealed changes mainly in the nuclei. In these, numerous electron-dense

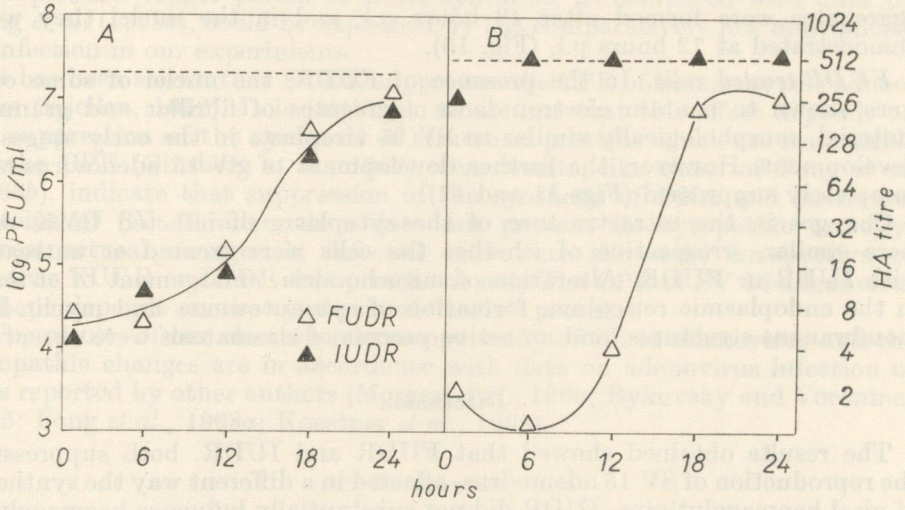

Fig. 3.

Influence on infectious virus reproduction (A) and haemagglutinin synthesis (B) of time interval after inoculation with adenovirus SV 15, at which FUDR or IUDR (100 μg/ml) was added to GMK cells

Abscissa: time interval between inoculation and addition of inhibitors (in hours)

------- Titre of infectious virus or haemagglutinins in untreated controls

areas appeared among the chromatin. The areas consisted of irregularly interwoven osmiophilic threads 20—30 Å thick. The size and density of the structure of this osmiophilic component varied considerably (Fig. 4). At 24 hours p.i., by the character of their morphological arrangement these structures resembled the viromatrix or viroplast characteristic of DNA-containing viruses. In these areas the number of granular components and ribosomes considerably increased; a part of the latter showed a linear arrangement. On the periphery and within the viroplasts, virions at different stages of maturation were detected (Fig. 5). The number of morphologically complete virions rapidly increased and at 48 hours p.i. they usually appeared in the form of crystalline aggregates (Figs 6 and 7). The growth of SV 15 adenovirus in GMK cells was accompanied by condensation and redistribution of chromatin as well as by an active process of clasmatosis. At 48 hours p.i. the continuity of the nuclear membrane was broken and evasion of the virions into the cytoplasm was observed.

IUDR-treated cultures. The changes observed in these cultures were similar

to those found in infected control cultures. The formation of viroplasts started at 16 hours p.i., but the rate of their development was considerably slower than in control cultures (Fig. 8). The formation of viroplasts was usually complete not before 48 hours p.i. (Fig. 9). As compared with control cultures, in which morphologically mature SV 15 virions were observed at 24 hours p.i. and crystalline arrangements of the virions at 48 hours p.i., in IUDR-treated cell cultures both the mature virions and crystal-like aggregates were formed after 48 hours p.i. and in the nuclei they were demonstrated at 72 hours p.i. (Fig. 10).

FUDR-treated cells. In the presence of FUDR, the nuclei of some cells were shown to contain electron-dense aggregates of fibrillar and granular material, morphologically similar to SV 15 viroplasts in the early stages of development. However, the further development of SV 15 adenovirus was completely suppressed (Figs 11 and 12).

Changes in the ultrastructure of the cytoplasm of infected GMK cells were similar, irrespective of whether the cells were treated or untreated with IUDR or FUDR. Alteration of mitochondria, enlargement of cisterns in the endoplasmic reticulum, formation of cytoautosomes and myelin-like membranous structures, and an active process of clasmatosis were observed.

Discussion

The results obtained showed that FUDR and IUDR, both suppressing the reproduction of SV 15 adenovirus, affected in a different way the synthesis of viral haemagglutinins. IUDR did not substantially influence haemagglutinin synthesis, whereas FUDR suppressed it. Electron microscopy revealed the formation of morphologically mature virions in the nuclei of IUDR-treated cells. FUDR completely suppressed the formation of adenovirions.

The difference in the effects of the inhibitors on haemagglutinin synthesis and infectious virus formation is connected with their different mechanisms of action. FUDR inhibits thymidilate synthetase and thus the synthesis of DNA, while IUDR (like bromodeoxyuridine) resembles deoxythymidine and is incorporated into viral DNA (see Prusoff, 1967).

Inhibiting the synthesis of viral DNA, FUDR prevents the formation of adenoviral structural proteins (Kjellen, 1962; Flanagan and Ginsberg, 1962). Fong *et al.* (1968*b*) showed that inhibition of the synthesis of SV 15 adenovirus DNA by cytosine arabinoside results in the inhibition of the synthesis of complement-fixing antigen. Our results indicate that DNA synthesis is also required for the formation of SV 15 adenovirus haemagglutinin.

Kjellen *et al.* (1963) demonstrated that, in the presence of bromodeoxyuridine, structural adenovirus proteins are synthesized and noninfectious virions formed. Similarly, the formation of noninfectious herpesvirus (Kaplan *et al.*, 1966) and vaccinia virus (Prusoff *et al.*, 1963) virions in the presence of IUDR was reported. The present results showed that IUDR-treated SV 15 adenovirus-infected GMK cells synthesized noninfectious virions. A study of the properties of such virions is of considerable interest. Recent studies (Tsetlin and Altstein, to be published) showed that noninfectious

virions of the oncogenic SA7 (C8) simian adenovirus produced in the presence of IUDR retain their oncogenicity for hamsters. Adenoviruses grown in the presence of IUDR may apparently serve as a new model for studies on the defectiveness of viruses and adenovirus oncogenesis.

According to our results, the synthesis of SV 15 viral DNA is accomplished in principle from 12 to 18 hours p.i. but apparently continues till the 24th hour p.i. The longer period of DNA synthesis, as compared with data by Fong *et al.* (1968*b*), could be explained by the comparatively low multiplicity of infection in our experiments.

It has been shown that FUDR suppresses the CPE of human type 5 adenovirus (Kjellen, 1962). It is justifiable to assume that this effect depends on the suppression of the synthesis of structural proteins which are responsible for the CPE (Ginsberg *et al.*, 1967). Our results, like those of Fong *et al.* (1968*b*), indicate that suppression of the synthesis of DNA and structural proteins of SV 15 adenovirus does not prevent its cytopathic activity. Ultrastructural changes in infected cells were the same irrespective of whether FUDR or IUDR was present or not. The CPE of SV 15 adenovirus perhaps depends on the synthesis of "early" proteins.

The results of our electron microscope study of SV 15 adenovirus and the cytopathic changes are in accordance with data on adenovirus infection of cells reported by other authors (Morgan *et al.*, 1960; Bykovsky and Voronina, 1965; Fong *et al.*, 1968*a*; Koestner *et al.*, 1968).

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Explanation of Electron Micrographs:

Fig. 4. SV 15-infected GMK cell, 16 hours p.i. Multiple nuclear inclusions appear as electron dense areas of various shapes (arrows). N, nucleus. × 15 000.

Fig. 5. SV 15-infected GMK cell, 24 hours p.i. Intranuclear sites of viral replication (arrows). Note electron transparency of virus-containing areas and their close proximity to electron-dense granular condensations. N, nucleus, × 30 000.

Fig. 6. SV 15-infected GMK cell, 48 hours p.i. Virus aggregates in the nucleus and in the cytoplasm (V). Note concentration of chromatin along the inner nuclear membrane (arrows). N, nucleus. × 30 000.

Fig. 7. SV 15-infected GMK cell, 48 hours p.i. Virus aggregate in the nucleus. × 70 000.

Fig. 8. SV 15-infected GMK cell treated with IUDR, 16 hours p.i. Multiple granular-fibrillar intranuclear inclusions appear as electron-dense areas of various shapes (arrows). N, nucleus. × 15 000.

Fig. 9. SV 15-infected GMK cell treated with IUDR, 48 hours p.i. Granular condensations of various electron density (arrows). N, nucleus. × 9 000.

Fig. 10. SV 15-infected GMK cell treated with IUDR, 72 hours p.i. Virus aggregate in the nucleus. × 120 000.

Fig. 11. SV 15-infected GMK cell treated with FUDR, 16 hours p.i. Granular condensations of various electron density (arrows). N, nucleus. × 30 000.

Fig. 12. SV 15-infected GMK cell treated with FUDR, 72 hours p.i. Granular condensations of various electron density (arrows). N, nucleus; *, vesiculated endoplasmic reticulum with amorphous material. × 30 000.