JOURNAL OF VIROLOGY, Feb. 1975, p. 253-258
Vol. 15, No. 2

Printed in U.S.A.

Stimulation of Adenovirus Replication in Simian Cells in the Absence of a Helper Virus by Pretreatment of the Cells with Iododeoxyuridine

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Received for publication 23 September 1974

Pretreatment of African green monkey kidney cells with 50 μg of 5'-iododeoxyuridine (IUdR) per ml can modify their susceptibility to the replication of human adenovirus type 7 in the absence of simian virus 40 (SV40) although this enhancement of adenovirus replication is not as efficient as that of the helper SV40 virus. Since the number of infectious centers remains unchanged after IUdR pretreatment whereas the burst size of virus from each infected cell increases, the IUdR appears to allow each infected cell to produce more virus. Cell DNA synthesis appears to be stimulated in IUdR pretreated cells infected with adenovirus 7, but the host cell DNA synthesized is small enough to remain in the Hirt supernatant fluid. The modification of susceptibility to adenovirus replication and the changed pattern of cell DNA synthesis is stable for at least two additional cell passages of the pretreated cells.

It is well established that human adenoviruses replicate poorly, if at all, in cells of simian origin unless a helper virus is present (22). In the abortive cycle, the adenovirus adsorbs, penetrates, and is uncoated. Synthesis of the early virus-specific T antigen is induced (5, 20), and virus DNA is produced at the same rate as in the productive cycle of replication (23, 25); no studies of the infectious nature of this DNA have been performed. There appears to be no detectable change in the production of mRNA in the abortive versus the productive cycles (3, 6) although this mRNA has never been used in in vitro protein synthesizing systems. Using indirect immunofluorescence tests, early workers could detect no synthesis of virus capsid antigen in the abortively infected cells (5, 20). However, with more specific reagents, small amounts of the various capsid antigens can be detected (7, 12).

Although the unrelated simian papovavirus SV40 has most frequently been used as helper virus to enhance the replication of human adenoviruses in monkey cells, simian adenoviruses such as SV15 and SA7 can also supply the helper function (2, 21). Temperature-sensitive mutants of SV40 have been used in attempts to determine which cistron of SV40 controls the enhancement of adenovirus replication. These studies (16, 17; Jerkofsky, manuscript in preparation) have determined that the SV40 function is an early one, prior to virus DNA synthesis; the SV40 functions not yet ruled out include the production of SV40 T antigen and the stimulation of host cell DNA synthesis. We have previously determined that a host cell component is involved in the interaction of human adenoviruses and SV40 in simian cells, since enhancement of adenovirus replication does not always occur in monkey cells in which SV40 is capable of efficient replication (15), and have suggested that the stimulation of host cell DNA synthesis by SV40 may be the critical step (16). Recent experiments by St. Jeor and Rapp (26, 27) have demonstrated that the susceptibility of a cell to infection with human cytomegalovirus may be increased by pretreatment with 5'-iododeoxyuridine (IUdR). We have used this technique to determine whether pretreatment of monkey cells with IUdR can render them susceptible to replication of adenovirus 7.

MATERIALS AND METHODS

Cells. Primary African green monkey kidney (GMK) cells were grown in medium 199 supplemented with 10% fetal calf serum, 10% tryptose phosphate broth, and 0.08% $NaHCO_3$. All media used contained 100 U of penicillin and 100 μg of streptomycin per ml. Primary human embryonic kidney cells were obtained from HEM and Flow Laboratories, Rockville, Md., and were grown in the same medium.

Viruses. The stock SV40 and adenovirus type 7 have been described previously (16).

Plaque assay. Adenovirus 7 yields were deter-

mined by plaque assay in human embryonic kidney cells and SV40 yields by plaque assay in GMK cells as described previously (16).

IUdR pretreatment and growth studies. Primary GMK cells were trypsinized and sedimented by low speed centrifugation. The cells were resuspended in medium containing various concentrations of IUdR or in drug-free medium and plated into 60-mm plastic petri dishes or 1-oz. glass prescription bottles. When a confluent monolayer of cells was obtained, the drug was removed. Virus at a multiplicity of infection of 3 to 5 PFU per cell was added and allowed to adsorb for 1 to 2 h at 37 C. The cell sheets were then washed two times with Tris-buffered saline to remove unadsorbed virus. Drug-free maintenance medium (Eagle medium with 0.23% $NaHCO_3$ and antibiotics) was then added and cultures were incubated at 37 C. At designated times, cultures were removed and plunged into an alcohol-dry ice bath. The samples were frozen and thawed two times, cell debris was removed by centrifugation, and portions were stored at −70 C until titrated for virus yield.

DNA studies. The Hirt technique (13) was used. Cultures were prepared and infected as described above. They were pulsed for 2 h at 22 h postinoculation with 10 μCi of tritiated thymidine per ml, specific activity 13 Ci/mmol (Schwarz/Mann).

Density gradient centrifugation analysis was performed with some samples. Sample (0.2 ml) was mixed with 3.8 ml of CsCl of a density of 1.745 g/cc and centrifuged to equilibrium for at least 60 h in a 40.3 rotor in a Beckman L350 centrifuge at 30,000 rpm at 20 C. Eight-drop fractions were collected by bottom puncture onto filter paper disks and radioactivity was measured as described above. Every tenth fraction was collected for determination of refractive index.

RESULTS

Effect of IUdR pretreatment on ability of GMK cells to replicate adenovirus 7 and SV40. Primary GMK cells were trypsinized and grown to confluency in medium containing various concentrations of IUdR or in drug-free medium. When cell monolayers had formed, the drug-containing medium was removed and adenovirus or adenovirus with SV40 was added. After virus adsorption and the removal of residual virus, maintenance medium containing no drug was placed on all cultures. Harvests were made at various times and assayed for virus yield. Table 1 illustrates the yield of adenovirus 7 from two such experiments. In the presence of SV40, the amount of adenovirus 7 produced is approximately the same in the drug-treated and untreated cultures. However, in the absence of SV40, as the concentration of IUdR present in the pretreated cells increases from 10 to 50 μg/ml, there is an increase in the amount of adenovirus 7 produced. Higher concentrations of the drug in the pretreated cells did not increase the yield of adenovirus. Therefore, in all further experiments, the cells were pretreated with 50 μg of IUdR per ml. At this concentration, the final virus yield is about 10-fold greater than the yield from control cells in the absence of SV40 but still about 10-fold lower than the yield from control cells in the presence of SV40. Therefore, it appears that pretreatment of GMK cells with IUdR can stimulate an enhancement of adenovirus replication but that this enhancement is not as efficient as that supplied by the helper SV40 virus.

Table 2 demonstrates the effect of pretreatment of GMK cells with IUdR on their ability to support the replication of SV40. At all concentrations of IUdR pretreatment there is a decrease in the efficiency of SV40 replication. This is true even at the 50-μg/ml concentration at which the replication of adenovirus 7 is maximally enhanced. However, this decrease in SV40 replication did not affect its ability to enhance the replication of adenovirus 7 (Table 1).

Infectious center assay. Infectious center assays were then performed to determine whether IUdR pretreatment changed the number of cells capable of producing infectious adenovirus. Drug pretreated and control GMK cells were prepared and infected as usual. Then, 4 to 6 h later, the cells were trypsinized, washed with Tris-buffered saline, and treated with

TABLE 1. *Effect of pretreatment with IUdR on the ability of GMK cells to replicate adenovirus 7 in the absence and presence of SV40*

Pretreatment (μg of IUdR per ml)	Yield of adenovirus 7 (PFU per culture)	
	No SV40	Plus SV40
None	3.4×10^4	2.5×10^6
10	4.5×10^4	5.0×10^6
50	2.5×10^5	3.0×10^6
None	6.0×10^4	4.0×10^7
50	3.5×10^6	5.5×10^7
100	1.9×10^6	1.9×10^7

TABLE 2. *Effect of pretreatment with IUdR on the ability of GMK cells to replicate SV40*

Pretreatment (μg of IUdR per ml)	Yield of SV40 (PFU per culture)
None	3.5×10^8
10	2.1×10^7
50	1.5×10^7
100	7.0×10^6

anti-adenovirus 7 antiserum to remove any remaining residual virus. The washed cells were then diluted and plated onto susceptible human embryonic kidney cells. The infected GMK cells were allowed to settle out overnight and then an agar overlay was added. The resulting plaques were counted and the number of plaques produced was divided by the number of plated GMK cells. Typical results are presented in Table 3. These results show that approximately one in every 40 GMK cells is capable of replicating adenovirus 7 so as to score as an infectious center. This is a minimal number since the cells tend to aggregate after repeated washing and centrifugation. There is no significant difference in the number of cells scoring as infectious centers under either condition of pretreatment or in the absence or presence of SV40; the threefold difference detected in the IUdR pretreated cells infected with adenovirus 7 is variable and is within the experimental error for this procedure. Therefore, it appears that IUdR does not significantly change the number of cells capable of releasing infectious adenovirus.

Determination of burst size. Other portions of the cells used in the previous experiment were used to determine the burst size of virus from each infected cell. In this case, the cells were allowed to incubate undisturbed for 48 h to produce maximal yields of adenovirus 7. The cells were then trypsinized and dilutions of infected cells were made. Virus was then released from the various dilutions of cells by two cycles of quick-freezing and thawing. The clarified supernatant fluids were then titrated for the amount of virus produced by various concentrations of infected cells. The results are presented in Table 4. In control GMK cells which received no drug pretreatment, each cell produced an average of 0.03 PFU of adenovirus 7. The amount of virus produced per cell was increased 10-fold in IUdR pretreated cells to 0.3 PFU of adenovirus 7 per cell. In the presence of SV40, there was no significant change in burst size with IUdR pretreatment. These results suggest that IUdR pretreatment does not change the number of monkey cells capable of replicating adenovirus 7 but that each infected cell is able to produce more virus.

Effect of IUdR pretreatment on the pattern of DNA synthesis. Since we had previously suggested that the stimulation of cell DNA synthesis by SV40 may be the important factor in the ability of SV40 to enhance adenovirus replication, we examined the patterns of DNA synthesis in IUdR pretreated infected cells. As described above, the infected cells were pulsed with tritiated thymidine for 2 h from 22 to 24 h post-inoculation and then chased with cold thymidine for an additional 2 h before the DNA was extracted by the Hirt technique. The most unexpected result was a 10-fold increase in tritiated thymidine incorporation in the Hirt supernatant from IUdR pretreated cells infected with adenovirus 7 (Table 5). No such increase was detected in the Hirt pellet from these cells.

This material was further analyzed by density gradient centrifugation to equilibrium in cesium chloride. Figure 1 shows the DNA profiles obtained from centrifugation of Hirt supernatant samples. In control, uninfected cells, a small peak of labeled DNA is detected at the density of cell DNA. In uninfected, IUdR pretreated cells, this peak is again evident and

TABLE 3. *Effect of pretreatment with IUdR on the ability of GMK cells infected with adenovirus 7 in the absence and presence of SV40 to score as infectious centers*

Virus	Pretreatment	PFU of adenovirus 7/no. of GMK cells plated
Adenovirus 7	None	1/40
Adenovirus 7	IUdR	1/125
Adenovirus 7 + SV40	None	1/43
Adenovirus 7 + SV40	IUdR	1/44

TABLE 4. *Effect of pretreatment with IUdR on the adenovirus burst size from GMK cells infected with adenovirus 7 in the absence and presence of SV40*

Virus	Pretreatment	PFU of adenovirus 7 released/no. of GMK cells disrupted
Adenovirus 7	None	0.03
Adenovirus 7	IUdR	0.3
Adenovirus 7 + SV40	None	20
Adenovirus 7 + SV40	IUdR	50

TABLE 5. *Effect of IUdR pretreatment on the pattern of DNA synthesis in GMK cells infected with adenovirus 7 in the presence or absence of SV40*

Virus	Hirt supernatant (counts/min)		Hirt pellet (counts/min)	
	No pretreatment	IUdR pretreatment	No pretreatment	IUdR pretreatment
None	581	480	3,916	1,463
Adenovirus 7	1,209	13,404	2,920	1,836
Adenovirus 7 + SV40	3,486	14,491	7,532	4,502
SV40	2,824	3,233	7,331	5,047

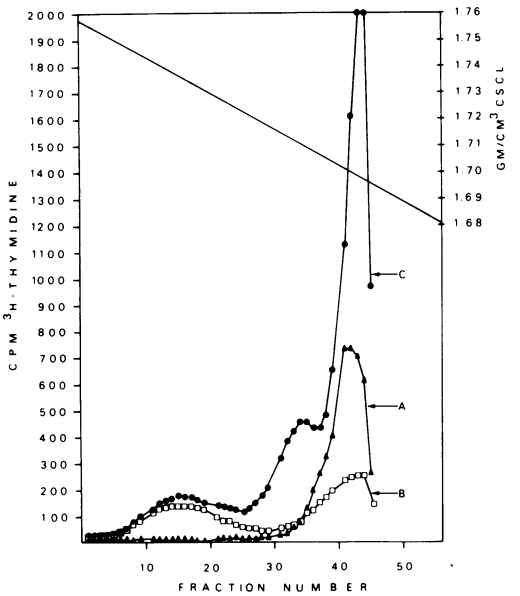

FIG. 1. *DNA profile of Hirt supernatant fractions spun to equilibrium in CsCl. A, Untreated GMK control cells; B, IUdR pretreated, uninfected cells; C, IUdR pretreated cells infected with adenovirus 7.*

there is a broad peak at a density where IUdR-substituted DNA should band. In the Hirt supernatant fraction from IUdR pretreated cells infected with adenovirus 7, the heavy IUdR-substituted peak is again visible as well as a peak banding at a density of 1.71 where adenovirus 7 virus DNA bands. There is also a greatly increased peak at the density of host cell DNA. The cell profiles obtained from untreated GMK cells infected with adenovirus 7 alone resembled those obtained with uninfected, untreated cells. Figure 2 shows the DNA profiles obtained from the Hirt pellet fractions. In the drug-free cells, there is only a host cell peak. The DNA profiles of the IUdR pretreated control and infected cells are almost identical. Therefore, it appears that there is a modification in the pattern of host cell DNA synthesis in the IUdR pretreated cells infected with adenovirus 7, but that the apparent stimulation of cell DNA synthesis is of a size of DNA small enough to remain in the Hirt supernatant.

Cell passage of IUdR pretreated cells. Experiments were also performed to determine how long IUdR pretreated cells remain more susceptible to adenovirus infection. Cells were pretreated with IUdR as usual. One half of the cultures were infected and labeled P-0. The other half were trypsinized and passed at a dilution of 1:2. When a cell monolayer was obtained, the cells were again trypsinized and passed at a dilution of 1:2. No additional IUdR was added after the original pretreatment. Figure 3 demonstrates the replication of adenovirus 7 in IUdR pretreated cells at P-0 and P-2 and in drug-free control cells. There is no difference in the replication of adenovirus 7 in the original IUdR pretreated cells and in cells which had undergone two additional cell passages. In both, the amount of adenovirus 7 produced is about

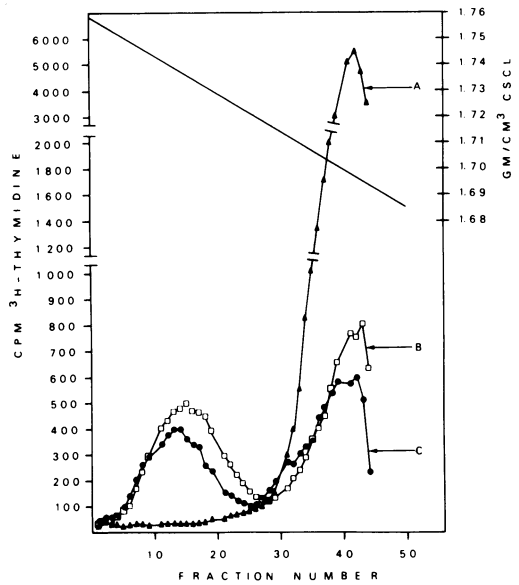

FIG. 2. *DNA profiles of Hirt pellet fractions spun to equilibrium in CsCl. A, Untreated GMK control cells; B, IUdR pretreated, uninfected cells; C, IUdR pretreated cells infected with adenovirus 7.*

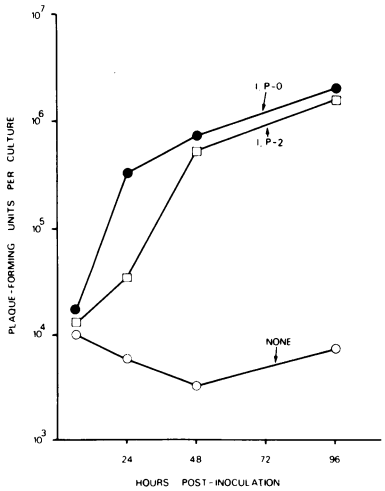

FIG. 3. *Replication of adenovirus 7 in GMK cells pretreated with 50 μg of IUdR per ml at the original passage (P-0) and after two additional cell passages in drug-free medium (P-2) and in control (none) GMK cells.*

100-fold greater than that produced in control cells. These results suggest that the initial IUdR pretreatment produced a change in GMK cell susceptibility to adenovirus replication which is stable for at least two additional cell passages. This observation has been reproduced in a number of experiments.

The pattern of DNA synthesis was also monitored at each passage level. Stimulation of tritiated thymidine uptake into the Hirt supernatant fraction of IUdR pretreated cells infected with adenovirus 7 was observed at all passages tested (Table 6); no change was detected in control and infected Hirt pellet fractions. After density gradient centrifugation to equilibrium, the DNA profiles were identical to those in Fig. 1 and 2. Therefore, it appears that the modification in the pattern of DNA synthesis was also detected for two additional passages of the pretreated cells.

TABLE 6. *Effect of cell passage of GMK cells pretreated with IUdR on the pattern of DNA synthesis in adenovirus 7 infected cells*

Passage level	Pretreatment	Virus	Hirt supernatant (counts/min)	Hirt pellet (counts/min)
P-0	None	None	183	2,136
	None	Adenovirus 7	628	1,333
	IUdR	None	212	1,121
	IUdR	Adenovirus 7	1,541	1,176
P-1	None	None	134	1,176
	None	Adenovirus 7	375	826
	IUdR	None	165	1,078
	IUdR	Adenovirus 7	1,990	1,117
P-2	None	None	80	584
	None	Adenovirus 7	222	468
	IUdR	None	113	1,179
	IUdR	Adenovirus 7	705	938

DISCUSSION

Results presented in this paper have demonstrated that pretreatment of GMK cells with IUdR can change their susceptibility to infection by human adenovirus 7, a result also obtained recently by S. P. Staal and W. P. Rowe (personal communication). There is an enhancement of adenovirus replication in the absence of additional helper virus. However, this enhancement is not as efficient as that observed when the cells are coinfected with the simian helper-virus SV40. IUdR pretreatment does not change the number of cells capable of producing infectious adenovirus but the amount of adenovirus produced by each infected cell is increased. Therefore, it appears that the IUdR pretreatment allows each infected cell to produce virus more efficiently.

There is a modification in the pattern of DNA synthesis in IUdR pretreated cells infected with adenovirus 7. Cell DNA synthesis is apparently stimulated but the newly made DNA is small enough to remain in the Hirt supernatant fraction (Table 5). Experiments are in progress to determine the size, homogeneity, and possible biological activity of this newly synthesized host cell DNA. Levine and Burger (18) have suggested that a modification in the pattern of host cell DNA synthesis could be the factor responsible for maintaining the transformed phenotype of SV40 transformed cells. Monkey cells transformed by SV40 can readily replicate human adenoviruses in the absence of added helper virus although the nontransformed parental cells could not (24). Therefore, it appears that the adenovirus enhancement function of SV40 is expressed in at least some transformed cells.

However, the exact mechanism of action of the IUdR in this system is unknown. IUdR has been shown to induce both RNA and DNA viruses from a latent state (1, 8–11, 19, 30) and to interfere with protein synthesis in normal cells (14, 28, 29). The requirement for actual incorporation into the DNA of IUdR pretreated cells in this system has yet to be determined. However, since our experiments are performed in primary GMK cells, all lots of cells are not identical. In an occasional lot, no enhancement of adenovirus replication was detected after IUdR pretreatment and there was no stimulation of thymidine uptake into the Hirt supernatant. At the present time, this is merely correlative. However, the persistence of the modified susceptibility phenotype and the modified pattern of DNA synthesis for several cell passages would suggest some sort of heritable change in the cells.

If the newly synthesized cell DNA is of homogeneous size and complexity, it is possible that only a small segment of the host genome must be replicated during adenovirus infection to render the cells susceptible. This segment could have been selectively reproduced after IUdR pretreatment and could account for the DNA found in the monkey cell adapting component (4) which is known to enhance adenovirus replication. SV40 and other helper viruses could efficiently replicate this segment of host DNA whereas the effect with IUdR treatment may be of a random nature and therefore less efficient. Since IUdR pretreatment also increases the susceptibility of cells to replication of human cytomegalovirus (26, 27), it would be of interest to determine if a similar modification in the

pattern of DNA synthesis is also observed in that system.

We have demonstrated that the modified phenotype is stable and heritable for at least two additional cell passages after IUdR pretreatment. Since primary cells have been used in these experiments, further passages are usually not feasible. To prove a heritable genetic change, a stable cell line may have to be employed so that the cells can be passed indefinitely.

ACKNOWLEDGMENTS

We would like to thank Sharon Faulkner and Sara Fetrow for excellent technical assistance.

This study was conducted under Public Health Service research grant no. 11647 from the National Cancer Institute.

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