stricted to fibroblasts cultured *in vitro;* and effects on other types of cells may have greater implications for this question.


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## Inhibition of Adenovirus Replication by 1-β-D-Arabinofuranosylcytosine.* (31100)

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Human adenovirus types 2, 5, 7, and 12 are unable to replicate in African green monkey (GMK) cell cultures(1-4). In these cells, the viruses are able to induce synthesis of adenovirus tumor (T) antigen but fail to induce synthesis of viral (V) capsid antigen (1). Co-infection with simian papovavirus 40 (SV40) potentiates replication of the adenoviruses(1-4) and results in induction of the adenovirus T and V antigens(1) as well as the synthesis of SV40 T and V antigens.

Previous studies in our laboratory indicated that 1-β-D-arabinofuranosylcytosine (cytosine arabinoside or ara-C) inhibited the replication of SV40(5,6) while allowing the synthesis of SV40 T antigen. Buthala(7) had suggested that ara-C does not inhibit replication of adenoviruses and when the antagonist was found to block the potentiation of adenovirus replication in GMK cells by SV40(8), it was concluded that SV40 T antigen was not responsible for the potentiation phenomenon. In order further to support such a conclusion, it was necessary to confirm that ara-C was not capable of inhibiting adenovirus replication. The results of quantitative studies designed to investigate the effect of ara-C on adenovirus replication in human cells are presented here.

*Materials and methods. Cell cultures and viruses.* Primary cultures of human embryonic kidney (HEK) cells were grown in one-oz bottles using Melnick-Hanks' (M-H) lac-

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talbumin hydrolysate medium(9) containing 10% fetal calf serum. Maintenance fluid for these cultures contained 2% fetal calf serum. Secondary cultures grown on 15 mm cover glasses in 60 mm plastic petri dishes were utilized for detection of antigens by immunofluorescence techniques(10). HeLa and KB cultures were grown in one-oz bottles in Eagle's medium with 10% calf serum; maintenance fluids contained 2% calf serum.

The viruses used as well as their preparation have been described previously(1). All the adenoviruses were human isolates propagated only in cells of human origin.

*Viral assays and immunofluorescence techniques.* Adenovirus titers were obtained in HEK cells by the plaque technique(1,11). Virus harvested from infected cultures was recovered following 3 cycles of quick-freezing and thawing of the cells and titers therefore represent total virus yield.

The detection of adenovirus T and V antigens by the indirect immunofluorescence technique has been described and the specificity of the serum used to detect the adenovirus T antigens has been demonstrated(1). Adenovirus T antigens were detected using sera from hamsters bearing transplanted adenovirus type 12 tumors; V antigens were detected by reacting infected HEK cultures with anti-adenovirus rabbit serum and anti-rabbit globulin labeled with fluorescein isothiocyanate.

*Results.* Human embryonic kidney monolayers were inoculated with adenovirus types 2, 7, and 12 at a multiplicity of infection of 2-6 plaque-forming units (PFU) per cell. The virus was allowed to adsorb to the cells for one hour at 37°C; the cultures were then washed twice with tris buffer (pH 7.4) to remove unadsorbed virus and flooded with maintenance medium. Half the cultures received 10 μg/ml of ara-C (kindly supplied by Upjohn Co., Kalamazoo, Mich.) added with the maintenance medium. Virus was harvested at 6, 24, 48, and 72 hours following inoculation of the cultures. Later harvests were not attempted because the drug-free but infected cultures exhibited extensive cytopathic effects (CPE) 72 hours following inoculation.

The results of such an experiment are presented in Fig. 1. All cultures showed an initial decrease in adenovirus titer. The yields of adenovirus types 2, 7, and 12 harvested 6 hours after inoculation were approximately the same in the presence and absence of the antagonist indicating that ara-C had no effect on eclipse of the viruses. Final viral harvests, taken when infected but drug-free cultures showed massive CPE (75-100% of the cells involved), yielded 100 to more than 1000 times the infectious virus than yields obtained from cultures treated with ara-C. The concentration of ara-C used in these experiments was not cytotoxic to the cells.

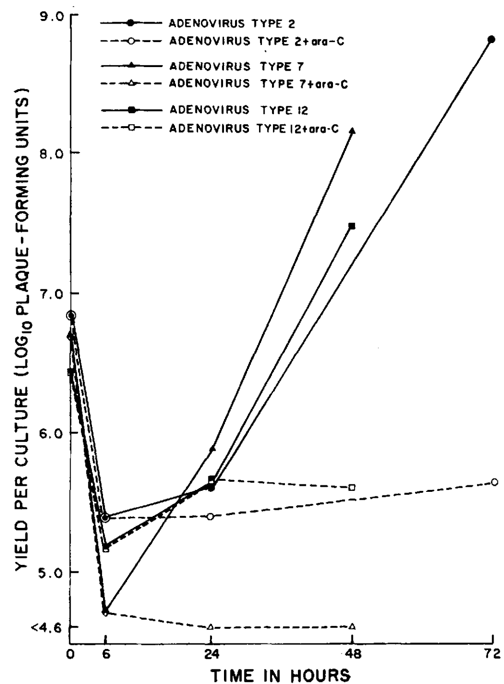


FIG. 1. Replication of adenovirus types 2, 7, and 12 in human embryonic kidney cells in absence and presence of ara-C (10 μg/ml).

Table I summarizes the results of an identical series of experiments carried out in KB and HeLa cells. Once again, ara-C inhibited the replication of adenovirus types 2, 7, and 12 when virus yields from drug-treated cultures were compared with yields from drug-free cultures. The titer of adenovirus type 12 obtained from drug-free HeLa and KB cultures, however, was lower than those obtained from drug-free HEK cultures.

Since a fraction of the input virus was recovered at all times in the presence and ab-

TABLE I. Replication of Adenoviruses in KB and HeLa Cell Cultures in Absence and Presence of Ara-C.

| | Titers (PFU per culture)* | | | | | |
|---|---|---|---|---|---|---|
| | KB cell cultures | | | HeLa cell cultures | | |
| Virus | No drug | Ara-C† | % Inhibition | No drug | Ara-C† | % Inhibition |
| Adenovirus type 2 | $2 \times 10^8$ | $2 \times 10^6$ | 99.9 | $>5 \times 10^6$ | $5 \times 10^4$ | >99 |
| " " 7 | $2 \times 10^7$ | $2 \times 10^5$ | 99 | $4 \times 10^5$ | $5 \times 10^3$ | 99 |
| " " 12 | $1 \times 10^4$ | $3 \times 10^3$ | 70 | $3 \times 10^4$ | $<5 \times 10^2$ | >98 |

* Cultures were harvested when 75-100% of the cells in the drug-free cultures exhibited cytopathic changes. None of the cultures was incubated for more than 72 hr after infection.
† 10 μg/ml of ara-C was added in the maintenance fluids.
PFU = plaque-forming units.

sence of ara-C, it was important to determine whether this virus was the result of replication or whether it represented residual virus. Adenovirus type 7 was diluted to $2.8 \times 10^4$ PFU per ml in normal maintenance medium and then incubated at 37°C in a stoppered test tube. Table II shows that after 72 hours of incubation, $2 \times 10^3$ PFU or 7% of the original virus survived. In comparison, Fig. 1 shows that in the presence of ara-C, 6% of the original virus survived; these results suggest that this persisting virus represents residual virus surviving thermal inactivation.

TABLE II. Inactivation of Adenovirus Type 7 at 37°C.

| Hr at 37°C | PFU/ml | % Surviving virus |
|---|---|---|
| 0 | $2.8 \times 10^4$ | 100 |
| 24 | $2.7 \times 10^3$ | 10 |
| 48 | $2.1 \times 10^3$ | 7 |
| 72 | $2.0 \times 10^3$ | 7 |

PFU = plaque-forming units.

The effect of ara-C on the induction of adenovirus specific antigens in HEK cells is shown in Table III. Adenovirus T antigens were detected 24 hours after infection; viral antigens were detected 48 hours after infection. In the absence of ara-C, both T and V antigens were synthesized. However, in the presence of 10 μg/ml of ara-C, V antigen was not detected. The induction of the adenovirus T antigen was not inhibited by this DNA antagonist.

Adenovirus T antigen induced by types 2, 7, and 12 was located in the nucleus of infected cells. The appearance of the antigen varied, with some cells containing small particulate deposits and others having large, globular masses of antigen (Fig. 2). Cultures incubated in the presence of ara-C contained fleck-like deposits in the nucleus but the particulate deposits and the globular masses failed to appear.

*Discussion.* The ability of ara-C to inhibit DNA synthesis has been demonstrated(12-14). Ara-C inhibits the reduction of cytidylic acid to 2′-deoxycytidylic acid but can itself be converted to its 5′-$PO_4$ esters. These nucleotides cannot be incorporated into DNA or RNA. The results of our studies indicate that adenovirus types 2, 7, and 12 are not able to replicate in the presence of ara-C. These results are not in agreement with an earlier report(7). This discrepancy may be due to the use of different strains of adenovirus, but this seems unlikely in view of the difficulty in ob-

TABLE III. Induction of Adenovirus Antigens in HEK Cells in Absence and Presence of Ara-C.

| | No drug | | Ara-C (10 μg/ml) | |
|---|---|---|---|---|
| Virus | T antigen* | V antigen† | T antigen* | V antigen† |
| Adenovirus type 2 | + | + | + | 0 |
| " " 7 | + | + | + | 0 |
| " " 12 | + | + | + | 0 |

* T antigen—adenovirus tumor antigen detected 24 hr post-inoculation.
† V antigen—adenovirus viral antigen detected 48 hr post-inoculation.
+ = presence of antigen detected by immunofluorescence method. 0 = antigen not detected by immunofluorescence method.

taining ara-C resistant mutants(15). Since Buthala(7) failed to present quantitative data, it is difficult to determine whether or not the adenoviruses used in his study had actually replicated or had merely caused CPE without replication. Since large amounts of residual virus survive incubation at 37°C for 72 hours and the amount of residual virus in the presence and absence of ara-C is the same, failure by Buthala(7) to demonstrate quantitatively the replication of adenoviruses in the presence of ara-C could explain the contradictory results obtained.

In view of the inhibitory effect of ara-C on adenovirus replication, previous interpretations that the synthesis of SV40 T antigen in mixed infections was not responsible for the potentiation phenomenon must be reexamined. The failure to observe adenovirus replication in cultures jointly infected with SV40 and blocked with ara-C may be the result of the inhibition of adenovirus itself. It is worth noting that the replication of an adenovirus type 7-SV40 (PARA) population(16) is also inhibited by ara-C(8).

Induction of adenovirus T antigens in the presence of ara-C indicates that DNA synthesis is not required for induction of the T antigen. Synthesis of viral antigen was inhibited by ara-C. These results are in agreement with earlier reports(17,18) that noted that 5-fluorodeoxyuridine inhibits the synthesis of adenovirus T but not V antigens and are similar to results obtained with SV40(5,6). The data thus far accumulated with adenoviruses and SV40 strongly suggest that synthesis of the T antigen of DNA viruses does not require synthesis of viral DNA but that progeny DNA is necessary for synthesis of viral capsid antigens. The appearance of only the fleck-like deposits in the presence of ara-C may indicate that synthesis of other early viral-induced antigens is inhibited.

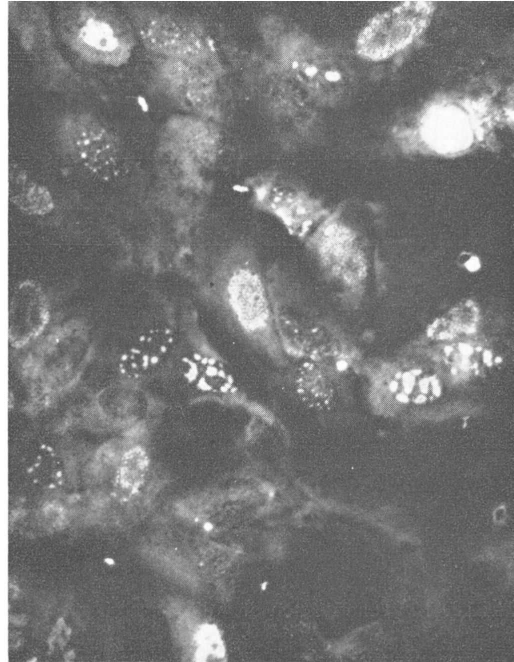

FIG. 2. Immunofluorescence photomicrograph of tumor antigen induced in human embryonic kidney cells by adenovirus type 7. Note intranuclear location of stippled and large globular masses of antigen. × 284.

*Summary.* The effect of 1-β-D-arabinofuranosylcytosine on replication of human adenovirus types 2, 7, and 12 in human embryonic kidney, KB, and HeLa cells was investigated. The results indicate that 10 μg/ml of ara-C inhibits replication of these adenoviruses in all 3 human cell types. Inhibition of synthesis of infectious virus ranged from 70 to greater than 99% when yields from infected cultures incubated in the presence of ara-C were compared to drug-free controls. Immunofluorescence studies of infected human embryonic kidney cells revealed that ara-C prevents synthesis of adenovirus viral antigens, but does not inhibit synthesis of adenovirus tumor antigens. However, appearance of the tumor antigen in the presence of ara-C was restricted to fleck- or fiber-like structures in contrast to the appearance of both flecks and globular masses in the absence of the drug.

ADDENDUM: Hollinshead and Huebner(19) have obtained similar results to those reported here with adenovirus type 12.

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## The Influence of Calcium on the Binding of Antithrombin by Antithrombin Inhibitor.* (31101)

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In our initial studies(1) it was shown that plasma antithrombin is normally held inactive by combination with an inhibitor. During clotting, a platelet factor is made available which binds the inhibitor, thereby releasing the antithrombin.

In the present studies, it was noted that the inhibitor requires calcium to bind antithrombin.

*Materials and methods.* 1. All materials and methods, other than those noted below, were previously described(1).

2. Calcium free plasma was prepared from intact, platelet free, nonanticoagulated plasma. The plasma was treated with 1 mg/ml disodium ethylenedinitrilotetraacetate, dihydrate (EDTA) and dialyzed at 5C against 3 changes of 0.9% NaCl. It was centrifuged clear and stored in plastic vials at —20C. These samples were stable for at least 3 weeks.

3. Calcium free human thrombin was prepared from thrombin(1) which was similarly treated with EDTA. It was dialyzed against 3 changes of veronal buffer and concentrated against polyvinylpyrrolidone in veronal buffer. The thrombin activity of the preparations was unchanged by the processing. Two ml volumes were stored in plastic vials at —20C. Preparations were exhausted in 2-3 weeks without change in potency.

4. All preparations were verified free of calcium by the method of Copp(2).

5. A standard thrombin activity curve was prepared using a sample of thrombin supplied by the Division of Biologics Standards, N.I.H., Bethesda, Md.

6. The method of study was described(1). In brief, it consisted of an incubation mixture composed of the reactants mentioned in the text. At stated intervals, an aliquot of the incubation mixture was added to a fibrinogen solution and the thrombin clotting time measured.

*Results.* Normal plasma, not deprived of its calcium, had minimal antithrombic activity (Fig. 1, curve 1A)(1). Similarly, calcium free plasma to which physiologic (M/360) calcium chloride was added, had minimal activity (Fig. 1, curve 1). The process of removal and readdition of the calcium chloride did not affect the activity.

In contrast, calcium free plasma had maximal antithrombic activity (Fig. 1, curve 2). With the addition of calcium to this plasma, suppression of antithrombic activity occurred, which varied inversely as the calcium concentration (Fig. 1, Fig. 3, curve 1). It is evi-

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