Inhibition of Synthesis of Herpes Simplex Virus Deoxyribonucleic Acid by a Carcinogenic Polycyclic Aromatic Hydrocarbon (36610)

R. J. GOLDBERG, J. J. DOCHERTY, AND F. RAPP

Department of Microbiology, College of Medicine, The Milton S. Hershey Medical Center of The Pennsylvania State University, Hershey, Pennsylvania 17033

In 1964, DeMaeyer and DeMaeyer-Guignard (5) reported inhibition of herpes simplex virus (HSV) replication by the polycyclic aromatic hydrocarbon (PAH) 7, 12-dimethylbenz[*a*]anthracene (DMBA). We confirmed this observation and demonstrated that both extracellular and cell-associated HSV yields were significantly reduced in infected rabbit kidney cultures incubated in the presence of DMBA (8). A mechanism to account for the decreased extracellular virus yields obtained in the presence of DMBA was postulated following the demonstration that HSV infectivity was inactivated following incubation of the virus with this compound in a cell free system. The noncarcinogenic PAH, anthracene (Ant), did not affect the infectivity of HSV in this system.

In this study, we have directed our attention to the intracellular events which occur following infection of cultured cells with HSV in the presence and absence of DMBA. Since this compound has repeatedly been shown to inhibit cellular DNA synthesis *in vivo* (11, 13) and *in vitro* (1, 2), attempts were made to determine changes in the pattern of DNA synthesis in cultured cells infected with HSV and maintained in the presence and absence of DMBA.

Materials and Methods. Rabbit kidney (RK) cultures. Primary cultures were grown in 8 oz prescription bottles and 60 × 15 mm plastic petri dishes as previously described (16). Secondary confluent monolayers were prepared from trypsinized cell suspensions obtained from primary RK cultures in 1 oz prescription bottles or on 75 × 25 mm microscope slides in 100 × 15 mm plastic petri dishes. The growth medium employed consisted of Eagle's basal medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), Fungizone (1 μg/ml), Mycostatin (10 units/ml) and 0.075% $NaHCO_3$ (closed cultures) or 0.23% $NaHCO_3$ (open cultures).

Virus. The 316-D strain of HSV type 2 was employed throughout this study. Methods for the propagation and plaque assay of this virus in primary RK cultures have been described (8).

Chemicals. The origin of the DMBA, Ant and dimethylsulfoxide (DMSO) employed in this study have been previously described (8). Stock solutions of the chemicals were prepared in DMSO at concentrations of 2 or 10 mg/ml. Final dilutions of the compounds were made by shaking appropriate aliquots of the stock solutions in medium for at least 2 hr at 37°. The final concentration of dimethylsulfoxide never exceeded 0.5% in the test systems. Appropriate measures were taken to protect the chemicals from exposure to light.

Autoradiography studies. Confluent secondary RK monolayers, grown on 75 × 25 mm microscope slides, were placed in 100 × 15 mm plastic tissue culture dishes and inoculated with 0.1 ml containing a quantity of HSV sufficient to effect a multiplicity of infection of 5 plaque forming units (PFU)/cell. The cultures were incubated at room temperature for 1 hr to allow virus adsorption and were then incubated in growth medium (10 ml/culture) containing 0.23% $NaHCO_3$ at 37° in an atmosphere of 5% CO_2 for 1 hr. At the end of this time, the medium was removed and replaced with either fresh growth medium or with medium containing the test chemical. After an additional 8 hr of incubation at 37°, the cultures were

pulsed with 0.5 μCi/ml of ^{3}H-thymidine (New England Nuclear, Boston, MA; sp act > 15 Ci/mmole) for 1 hr at 37°. The cultures were then washed with 0.025 *M* tris(hydroxymethyl)aminoethane (Tris) saline (pH 7.4), fixed for 15 min (with 20 parts 95% ethanol, 2 parts formalin, and 1 part glacial acetic acid), washed consecutively with 70% ethanol and 95% ethanol and air dried. Following exposure of the cultures to Kodak NTB2 nuclear emulsion (diluted 1:2 in distilled water) for 7 days at 10°, the slides were developed for 3 min at 19° in Kodak D19 (diluted 1:2), washed in tap water and fixed for 15 min in Kodak fixer. The cells were then stained with hematoxylin and the percentage of labeled nuclei was determined on replicate samples for each treatment after correction for background. A minimum of 500 nuclei were counted on each slide.

Isopyknic centrifugation in cesium chloride. Confluent secondary RK monolayers, in 1 oz prescription bottles, were infected with HSV and treated with the test chemicals in a manner similar to that described for the autoradiography studies with the exception that each culture received a total volume of 5 ml of growth medium. At 10 hr postinfection, 3 ml of the test medium was removed from each culture and ^{3}H-thymidine was added to the remaining 2 ml of culture fluid to a final concentration of 5 μCi/ml. The cultures were incubated at 37° for an additional hour followed by rapid freezing in dry ice to stop the incorporation of the isotope.

The cultures were frozen and thawed two times and the cells were scraped from the glass into the culture fluids with a rubber policeman. The contents of each culture were placed in glass tubes and digested for 6 hr at 37° with 0.2% Sarkosyl NL30 (Geigy Chemical Corp.), 0.02 *M* ethylenediaminetetraacetic acid and 0.2% pronase (Calbiochem, B grade). The pronase was prepared as a stock solution of 30 mg/ml in 0.1X SSC (0.015 *M* sodium chloride, 0.0015 *M* sodium citrate, pH 7.3) and was preincubated at 37° for 2 hr at 37° before use. Following digestion, .02 ml aliquots of each sample were mixed with 3.8 ml of cesium chloride (ρ = 1.745 g/cm^3) diluted in 0.1X SSC and centrifuged at 30,000 rpm for 65 hr at 17° in a Beckman L2-65B ultracentrifuge (40.3 fixed angle rotor). Fractions were collected by bottom puncture onto Whatman No. 3 filter paper discs, air dried and washed consecutively at room temperature with 5% trichloroacetic acid (TCA), 95% ethanol and ethyl ether. Each filter was then placed in 10 ml of toluene–Omnifluor (New England Nuclear) and the amount of radioactivity was determined by counting in a Beckman LS-233 liquid scintillation counter. Selected fractions were also collected for measurement of refractive indices in a Bausch and Lomb refractometer.

Results. Autoradiographic analysis of the incorporation of ^{3}H-thymidine by rabbit kidney cells following infection with herpes simplex virus type 2. Initial experiments were performed to determine the pattern of incorporation of ^{3}H-thymidine in RK cultures following infection with HSV-2. Replicate RK cultures were infected with HSV-2 as indicated in Materials and Methods and were pulsed with ^{3}H-thymidine (0.5 μCi/ml) for 1 hr at 37° at various intervals postinfection. Uninfected RK cultures were treated in a similar manner. Following processing for autoradiographic analysis, the cells were examined microscopically for the presence of the silver grains indicative of incorporation of the isotope.

As expected, incorporation of the ^{3}H-thymidine was limited to the nuclei of RK cells in both infected and uninfected cultures and reflects the singularity of this structure as the site of HSV DNA synthesis (18). Quantitative analysis of the incorporation of ^{3}H-thymidine revealed that as early as 6 hr after infection, uptake of the labeled compound by infected cultures greatly exceeded that observed in control cultures. Over 50% of the nuclei were labeled in infected cultures pulsed after 7 hr postinfection whereas relatively few cells in uninfected cultures incorporated the isotope at all time periods tested (Table I). The progression of virus induced cytopathology precluded accurate enumeration of labeled nuclei in cultures infected for periods in excess of 11 hr.

TABLE I. Incorporation of Tritiated Thymidine by Rabbit Kidney Cells Following Infection with Herpes Simplex Virus Type 2.

Time of addition of ^{3}H-thymidine (hr postinfection)		% Labeled nuclei
6	+HSV	34
	−HSV	4
7	+HSV	28
	−HSV	7
8	+HSV	52
	−HSV	—
10	+HSV	54
	−HSV	4
11	+HSV	58
	−HSV	8

Effect of DMBA on DNA synthesis in rabbit kidney cells infected with herpes simplex virus type 2. A. Autoradiography studies. Autoradiographic studies were conducted to test the effect of DMBA on DNA synthesis in RK cells following infection with HSV-2. The noncarcinogenic hydrocarbon, Ant, was also tested. Replicate RK cultures were challenged with HSV-2 and treated with the chemicals as described in Materials and Methods. The percentage of labeled nuclei was determined following processing of the cultures for autoradiographic analyses.

The results of these studies are presented in Table II. Neither the chemical solvent DMSO, nor the noncarcinogenic hydrocarbon Ant diminished the number of labeled nuclei in HSV-2 infected cultures. However, a marked reduction in the percentage of labeled nuclei was noted at all concentrations of DMBA tested with the exception of 0.01 μg/ml.

B. Isopyknic centrifugation studies. Results of the autoradiographic studies suggested that the depression in DNA synthesis in HSV-2 infected RK cells following exposure to DMBA was the result of an inhibition of virus-specific DNA synthesis. Samples from DMBA treated and untreated HSV-2 infected cultures were subjected to isopyknic centrifugation in cesium chloride to determine the species of DNA synthesis inhibited in this system. Confluent RK monolayers in 1 oz bottles were infected with HSV-2, treated with DMBA, Ant, or the chemical solvent, DMSO, and were pulsed with ^{3}H-thymidine as described in Materials and Methods. Uninfected cultures which were not exposed to the chemicals were included to follow the changes in DNA synthesis affected by virus infection.

The results of these studies are presented in Fig. 1. The small amount of cellular DNA synthesis ($\rho = 1.699$) in uninfected RK monolayers was virtually eliminated following infection with HSV-2. This depression in cellular DNA synthesis following infection of cultured cells with HSV is consistent with previous reports (4, 18). No such inhibition resulted from treatment of replicate infected cultures with the noncarcinogenic compound, Ant (10 μg/ml). However, a marked inhibition of virus DNA synthesis ($\rho = 1.728$) was observed in infected cultures which were treated with DMBA (10 μg/ml). In similar experiments, no inhibition of incorporation of tritiated thymidine into TCA insoluble material was observed in HSV infected cultures exposed to 0.01 μg/ml DMBA while a concentration of 20 μg/ml restricted uptake of the isotope to approximately 50% of that observed in DMSO treated infected cultures. The results of these experiments confirm those obtained in the autoradiography studies and clearly demonstrate the ability of DMBA to inhibit HSV-2 DNA synthesis in RK cells.

TABLE II. Effect of Various Concentrations of 7,12-Dimethylbenz[*a*]anthracene on the Incorporation of ^{3}H-Thymidine by Rabbit Kidney Cells Following Infection with Herpes Simplex Virus Type 2.

Treatment of rabbit kidney cells	% Labeled nuclei
Uninfected	3
HSV	58
HSV + DMSO (0.5%)	61
HSV + Ant, 20 μg/ml	62
HSV + DMBA, 20 μg/ml	28
10 μg/ml	36
1 μg/ml	44
0.01 μg/ml	56

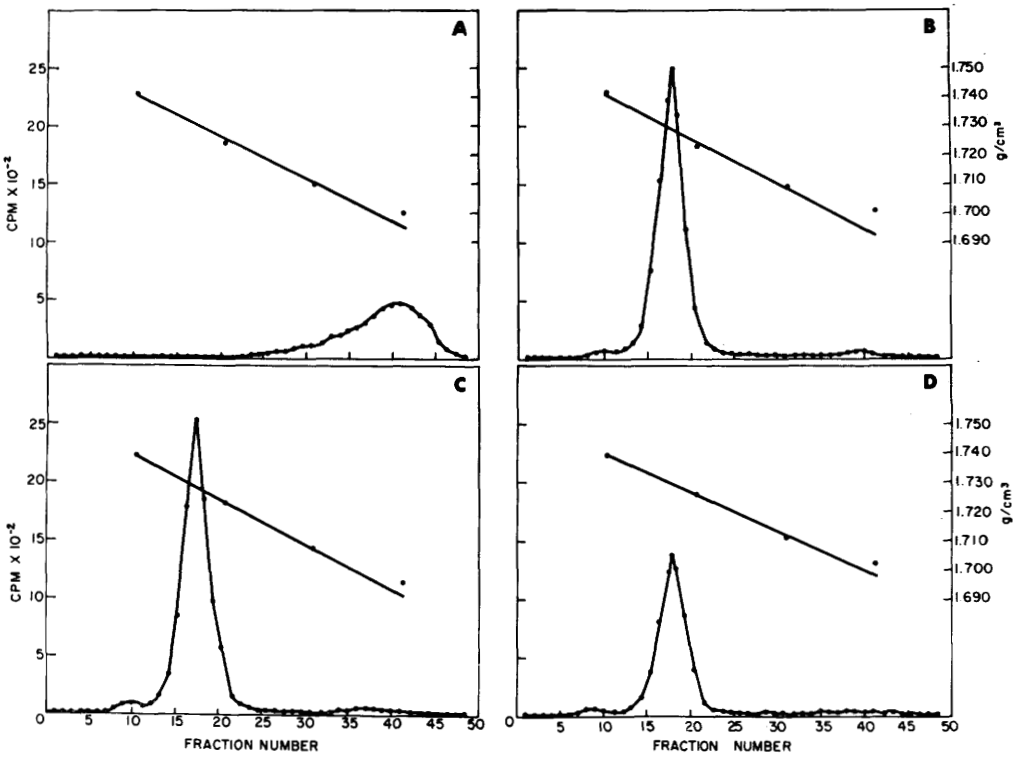

FIG. 1. Analysis of the incorporation of tritiated thymidine into acid insoluble material in rabbit kidney cells by isopyknic centrifugation in cesium chloride. (A) Uninfected cultures; (B) HSV-2 infected cultures treated with DMSO (0.5%); (C) HSV-2 infected cultures treated with Ant (10 μg/ml); (D) HSV-2 infected cultures treated with DMBA (10 μg/ml).

Cytotoxicity studies. The possibility was considered that the inhibition of DNA synthesis in DMBA treated cultures resulted from a nonspecific cytocidal interaction between the chemical and the infected cells. This hypothesis was tested in experiments in which viable cell counts were performed on untreated herpes infected cultures and on infected cultures treated with DMBA. Secondary RK cultures were infected and treated with chemicals (10 μg/ml) as described for the autoradiography experiments with the exception that no ^{3}H-thymidine was added during the final hour of incubation. Following termination of the experiment, the cells were washed with Tris–saline, suspended by trypsinization and viable cell counts were performed on replicate cultures using the vital dye, trypan blue.

Microscopic examination of HSV-2 infected cultures showed no morphologic differences between untreated RK cells and cells treated with DMBA, Ant or DMSO. The results depicted in Table III show that neither DMBA nor Ant at a concentration of 10 μg/ml produced a reduction in the number of viable cells in HSV-2 infected RK cultures. In similar experiments, DMBA was shown to be noncytocidal in this system when tested at concentrations as high as 20 μg/ml.

Discussion. The PAH's are a group of

TABLE III. Effect of Polycyclic Aromatic Hydrocarbons on the Viability of Rabbit Kidney Cells Following Infection with Herpes Simplex Virus Type 2.

Chemical added (10 μg/ml)	Viable cell no. (× 10^5)
None	6.1
Ant	6.4
DMBA	6.8

chemically related compounds, many of which possess the potential to induce oncogenic transformation in various animal cells *in vivo* and *in vitro*. The exact mechanism of their carcinogenicity is unknown although the association of these hydrophobic and lipophilic compounds with lipids, proteins and nucleic acids has been demonstrated (3, 7, 9, 10, 12). In addition, DMBA, one of the more potent carcinogenic PAH's, has been shown to be an effective inhibitor of cellular DNA synthesis *in vivo* and *in vitro* (1, 2, 11, 13).

Relatively little information is available concerning the interactions of chemical carcinogens and animal viruses at the molecular level. DMBA was found to bind to poliovirus type 2 *in vitro*. However, no significant uptake of this compound by either vaccinia virus or by the isolated nucleic acids from these two viruses was demonstrated (15). In addition, direct incubation of virus and carcinogen was found to inactivate the virus (14).

The selective inhibition of DNA virus replication and suppression of interferon synthesis by doses of carcinogenic PAH's which did not influence RNA virus replication has been reported (5, 6). No such effects were noted when anthracene and three other structurally related but noncarcinogenic PAH's were employed. It was suggested that the effects were most probably the result of a combination of the carcinogenic hydrocarbons with either cellular or virus DNA followed by the subsequent prevention of the expression of genetic information. This hypothesis is indeed supported by the available literature in which the binding of DMBA by cellular (3, 7, 12) and bacteriophage (14) DNA has been demonstrated.

The observed inhibition of DNA synthesis in HSV infected cultures along with the results of our previous studies which demonstrated the direct inactivation of herpesvirus infectivity by DMBA have revealed at least two levels at which this chemical carcinogen may induce a defective cycle of virus replication. These results are especially interesting in light of the relationship between virus induced neoplasia and defects in the replicative cycle of oncogenic viruses (17).

Summary. An inhibition of HSV DNA synthesis was observed in infected RK cells following treatment with the carcinogenic polycyclic aromatic hydrocarbon, DMBA. No such effect was observed in replicate cultures treated with the related but noncarcinogenic chemical, anthracene. The compounds were without cytocidal effects in the experimental system employed at the concentrations tested.

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