Differential Effect of 7,12-Dimethylbenz[*a*]anthracene on Infectivity of Herpes Simplex Virus Type 2[1] (35258)

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Herpes simplex virus (HSV) has been subdivided into 2 groups based on the area from which the viruses were isolated; either oral or genital. Although the basis for grouping oral isolates as type 1 and genital isolates as type 2 is serological (1, 2), the 2 types differ in other biological characteristics. These differences include pock formation on the chorioallantoic membrane of embryonated hen eggs, plaque formation in chick embryo cell cultures and neurovirulence in mice (3, 4).

A variety of studies have shown an association of herpesviruses with numerous types of cancer (5–11), and epidemiological studies have suggested a link of HSV-type 2 to human cervical carcinoma (12). In addition, it is known that certain chemicals can act as carcinogenic agents. One of the most potent chemical compounds with this property is 7,12-dimethylbenz[*a*]anthracene (DMBA).

In our studies we have noted that DMBA, a carcinogenic polycyclic aromatic hydrocarbon, has a preferential effect on HSV-type 2 rather than on HSV-type 1. Our results have shown HSV-type 2 is more sensitive to DMBA than type 1 as measured by inactivation of infectivity.

Materials and Methods. Primary rabbit kidney (RK) cultures. Monolayer cultures were grown in 1-oz prescription bottles and 60 × 15-mm plastic petri dishes as previously described (13). The medium used for cell cultivation consisted of 10% bovine serum in Eagle's basal medium with 0.075% $NaHCO_3$ (closed cultures) or 0.23% $NaHCO_3$ (open cultures). Each ml of the medium was additionally supplemented with 100 units of penicillin, 100 μg of streptomycin, 1 μg of Fungizone, and 10 units of Mycostatin.

Virus. All herpes simplex virus (HSV) strains used in this study were obtained from Dr. William Rawls, Baylor College of Medicine, Houston, Texas. Virus stocks were prepared in primary RK cells in 8-oz bottles. Infected cultures were harvested following incubation for 2 to 3 days at 37° at which time approximately 75% of the cells exhibited cytopathic changes. The infected cultures were ruptured by 2 cycles of alternate freezing and thawing, clarified by centrifugation at 1500 rpm for 10 min, and the supernatant fluid was collected, quick-frozen, and stored at −65° until used.

Plaque assay. HSV was titrated by a modification of the plaque assay technique in RK cells under a methylcellulose overlay as previously described (13). Virus samples were diluted in 0.025 *M* tris(hydroxymethyl) aminoethane (Tris) saline (pH 7.4) containing 3% bovine serum and antibiotics. The virus, in 0.1-ml amounts, was inoculated into each of 2 primary RK cell cultures in 60-mm plastic dishes and adsorbed for 1 hr at room temperature with intermittent manual rotation. Each plate was then overlayed with 5 ml of Eagle's basal medium supplemented with 0.5% methylcellulose, 5% bovine serum, antibiotics and 0.23% $NaHCO_3$. Following 4 days of incubation at 37° in an atmosphere of 5% CO_2, the cultures were stained with a 1:7500 solution of neutral red in Tris saline. The plaques were counted 1 hr after the addition of the dye.

Virus growth studies. Primary RK cultures, grown in 1-oz prescription bottles, were drained and inoculated with 0.1-ml volumes of HSV containing 10^6 plaque-forming units (PFU). The virus was allowed to adsorb for 1 hr at room temperature with frequent rota-

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tion to insure uniform distribution of the virus inoculum. The cultures were then washed 3 times with Eagle's basal medium containing 10% fetal calf serum, antibiotics, and 0.075% $NaHCO_3$ to remove unattached virus, and overlayed with 5 ml of the same nutrient medium. Polycyclic aromatic hydrocarbons were added to the overlay medium as indicated. The bottles were incubated at 37° and sampled for production of virus at various intervals after infection. Extracellular virus was obtained by harvesting the supernatant fluids from infected cultures and rinsing the monolayers 2 times with nutrient medium. The fluids from each culture were pooled and clarified by centrifugation at 1500 rpm for 10 min at room temperature. Cell-associated virus was obtained by adding 5 ml of nutrient medium to the washed cell sheets and freezing and thawing the cultures 2 times to release intracellular virus.

Chemicals. DMBA was purchased from Eastman Organic Chemicals, Rochester, New York. Anthracene (Ant) was obtained from K & K Laboratories, Inc., Plainview, New York. Dimethylsulfoxide (DMSO) was purchased from Matheson Coleman and Bell, Norwood, Ohio. Stock solutions of DMBA or Ant were prepared with DMSO as solvent. Further dilutions of these compounds were made in Tris buffer or Eagle's nutrient medium where indicated to produce a colloidal solution.

Results. Infection of RK cells in the presence of DMBA. Initial studies were performed to study the effect of this chemcial carcinogen on the growth of a type 2 HSV. Primary RK cells, in 1-oz bottles, were infected with the 316D strain of HSV-type 2 as indicated in Materials and Methods. Infected cultures were incubated in the presence or absence of DMBA or as control, the noncarcinogenic Ant, at a final concentration of 10 μg/ml. Additional controls included infected cultures incubated in the presence of a concentration of DMSO equivalent to that used as solvent for DMBA or Ant.

The results of this experiment are presented in Table I. It is evident that comparable virus titers were obtained in untreated and in Ant- or DMSO-treated RK cultures. The data also show that extracellular and cell-associated HSV yields were significantly reduced in infected RK cultures incubated in the presence of DMBA. The appearance of infectious extracellular virus was virtually eliminated in DMBA-treated cultures while the limited titers of cell-associated virus measured in these cultures at 24 hr decreased in the presence of this compound.

TABLE I. The Effect of Noncarcinogenic and Carcinogenic Polycyclic Aromatic Hydrocarbons on Intracellular and Extracellular HSV-type 2.

Treatment	Titer of herpes simplex virus (PFU/ml); time in hr		
	0	24	48
Extracellular virus			
None	8.5×10^2	1.3×10^5	1.8×10^5
DMBA[a]	2.5×10^1	$<10^1$	5.0×10^1
Ant[a]	4.7×10^2	6.2×10^4	2.1×10^5
DMSO[b]	7.8×10^2	5.5×10^4	2.9×10^5
Cell-associated virus			
None	2.3×10^3	1.4×10^6	1.2×10^6
DMBA	4.6×10^2	1.3×10^4	1.0×10^2
Ant	1.7×10^3	5.0×10^5	5.3×10^5
DMSO	2.4×10^3	1.9×10^6	8.0×10^5

[a] After virus adsorption the chemicals were added at a concentration of 10 μg/ml of growth medium.

[b] Dimethylsulfoxide was used as the diluent for the chemicals.

Effect of DMBA on HSV infectivity. The preceeding results suggested that the decreased HSV yields obtained in DMBA-treated cultures may have resulted from the direct inactivation of the infectivity of this virus by the chemical. This hypothesis was tested by measuring the effect of DMBA, Ant, and DMSO on the infectivity of HSV at 37°. Virus dilutions were made in Tris saline and the chemicals, prepared as stock solutions in DMSO, were added to the diluted virus to give the indicated final concentrations. The test mixtures were sampled following 0-, 45-, and 90-min incubation at 37° and immediately assayed on RK cells as indicated in Materials and Methods.

The data presented in Table II demonstrate the inactivation of HSV infectivity by DMBA at concentrations as low as 10 μg/ml

TABLE II. The Effect of Varying Concentrations of Noncarcinogenic and Carcinogenic Aromatic Hydrocarbons on the Infectivity of HSV-type 2.

Treatment (μg/ml)		Titer of herpes simplex virus (PFU/ml); time in min			Reduction of infectivity at 90 min (%)
		0	45	90	
None		4.2×10^5	2.5×10^5	2.6×10^5	38
DMSO[a]		3.4×10^5	2.4×10^5	2.6×10^5	24
Ant,	100	3.6×10^5	2.6×10^5	2.8×10^5	22
	50	2.8×10^5	2.6×10^5	2.2×10^5	21
	10	2.9×10^5	2.1×10^5	3.3×10^5	0
DMBA,	100	2.3×10^5	1.7×10^3	$<10^1$	>99
	50	2.1×10^5	2.3×10^4	2.1×10^3	99
	10	1.9×10^5	1.3×10^5	6.2×10^4	67

[a] Dimethylsulfoxide was used as the diluent for the chemicals.

over the 90-min test period. Additionally, a direct relationship was observed between the concentration of DMBA employed and the extent of virus inactivation. Exposure to 1 μg/ml of DMBA did not inactivate HSV after 45 min. In contrast, both Ant, at all concentrations tested, and DMSO were without appreciable effect (over control levels) on the stability of this virus.

Effect of temperature on inactivation of HSV by DMBA. Experiments were conducted to determine whether the inactivation of HSV by DMBA was temperature dependent. In addition, it was decided to study the comparative effect of this chemical on HSV-types 1 and 2. The Seibert strain of HSV-type 1 and the 316D strain of HSV-type 2 were mixed with DMBA, as previously, at a final concentration of 50 μg/ml. Virus controls were diluted in DMBA-free Tris saline. Replicate samples were incubated at 37, 25, and 1° (ice bath). The reaction mixtures were sampled following initial mixing of virus and chemical (0 min) and following incubation for 100 min at the respective temperatures. Samples were immediately titrated in prima-

TABLE III. The Effect of Temperature on the Inactivation of HSV-type 1 and HSV-type 2 by DMBA.

Virus type and treatment	Temp (°C)	(PFU/ml)		Reduction of infectivity (%)
		0 min	100 min	
HSV-type 1 control	1	4.1×10^6	2.9×10^6	29
	25	4.5×10^6	4.4×10^6	2
	37	4.6×10^6	4.9×10^6	0
+ DMBA[a]	1	3.7×10^6	2.3×10^6	38
	25	3.0×10^6	1.4×10^6	53
	37	3.2×10^6	2.4×10^5	93
HSV-type 2 control	1	2.5×10^5	2.1×10^5	16
	25	1.8×10^5	2.1×10^5	0
	37	2.7×10^5	2.5×10^5	7
+ DMBA[a]	1	1.1×10^5	4.3×10^4	61
	25	1.2×10^5	1.3×10^4	89
	37	1.5×10^5	1.0×10^2	>99

[a] The virus was mixed with 50 μg of DMBA/ml and incubated at 37°, 25°, or at ice bath temperature for 100 min.

ry RK cultures.

Maximum inactivation of HSV by DMBA occurred at 37° (Table III). Decreased levels of virus inactivation in the presence of DMBA were observed with a reduction in temperature of the reaction mixtures.

Surprisingly, the data in Table III also revealed that HSV-type 2 was more susceptible to inactivation by DMBA than HSV-type 1 at all temperatures tested.

Susceptibility of HSV-type 1 and HSV-type 2 to DMBA. The observation that HSV-type 2 was more susceptible to inactivation by DMBA than HSV-type 1 was investigated on a larger scale. The stability of 4 strains of HSV-type 1 and 9 strains of HSV-type 2 to DMBA at a final concentration of 50 μg of DMBA/ml at 37° for 100 min was then established.

The results of this experiment are summarized in Table IV. All 4 strains of HSV-type 1 exhibited a reduction in titer of 91% or less when incubated at 37° with DMBA. All 9 strains of HSV-type 2 tested were inactivated by 98% or more. Control studies revealed the absence of significant inactivation of all viruses by the noncarcinogenic polycyclic aromatic hydrocarbon, Ant (at 50 μg/ml) or DMSO when tested under similar conditions. This data, therefore, points to the selectivity of DMBA for the inactivation of the infectivity of HSV-type 2 as compared to its effect on HSV-type 1.

Discussion. De Maeyer and De Maeyer-Guignard (14) originally reported the inhibition of HSV replication by the polycyclic aromatic hydrocarbon DMBA. Our study of the interaction of herpes simplex virus and

TABLE IV. The Effect of DMBA on HSV-type 1 and HSV-type 2.

HSV type	Strain	Treatment	(PFU/ml) 0 min	(PFU/ml) 100 min	Reduction of infectivity (%)
1	Hill	Control	7.3×10^5	6.6×10^5	10
		DMBA[a]	7.0×10^5	7.1×10^4	90
1	Haywood	Control	3.0×10^5	4.3×10^5	0
		DMBA	2.3×10^5	2.0×10^4	91
1	Edna	Control	4.2×10^5	4.7×10^5	0
		DMBA	4.2×10^5	5.2×10^4	88
1	Seibert	Control	2.7×10^5	3.2×10^5	0
		DMBA	3.7×10^5	1.4×10^5	62
2	324	Control	1.1×10^4	1.1×10^4	0
		DMBA	1.2×10^4	6.0×10^1	>99
2	198	Control	5.6×10^4	7.7×10^4	0
		DMBA	5.5×10^4	1.0×10^1	>99
2	333	Control	1.0×10^5	9.0×10^4	10
		DMBA	6.0×10^4	7.0×10^1	>99
2	316D	Control	1.2×10^4	1.5×10^4	0
		DMBA	1.4×10^4	7.0×10^1	>99
2	332	Control	1.8×10^4	9.0×10^3	50
		DMBA	1.3×10^4	2.4×10^2	98
2	French	Control	4.2×10^3	5.8×10^3	0
		DMBA	4.3×10^3	$<10^1$	>99
2	186	Control	4.5×10^3	4.8×10^3	0
		DMBA	1.2×10^4	5.0×10^1	>99
2	327	Control	4.9×10^3	4.1×10^3	16
		DMBA	5.6×10^3	5.0×10^1	99
2	307	Control	2.1×10^4	2.0×10^4	5
		DMBA	1.9×10^4	2.0×10^2	99

[a] HSV was mixed with 50 μg of DMBA/ml and incubated at 37° for 100 min.

polycyclic aromatic hydrocarbons revealed that HSV-type 2 is more sensitive to DMBA than HSV-type 1 as measured by loss of infectivity following direct exposure to the compound. The reaction was a direct *in vitro* effect free of a cell-mediated system and was temperature and concentration (DMBA) dependent. The noncarcinogenic polycyclic aromatic hydrocarbon, anthracene, had no perceptible effect on either of the herpes types.

The polycyclic aromatic hydrocarbons are hydrophobic and lipophilic and have been shown to associate to varying degrees with lipids, proteins, and/or nucleic acids (15–18). The lipid association appears to be less stable than the more stable association of the chemicals with proteins or nucleic acids. As HSV contains all the chemical constituents (19) that the polycyclic aromatic hydrocarbons are known to react with, it is difficult to clearly establish the mechanism of inactivation of HSV by DMBA. A reaction of DMBA at the level of the lipoprotein envelope of HSV could lead to loss of infectivity. An interaction of chemical and virus at this point might inactivate or alter the overall charge on the virus leading to inhibition of specific attachment mechanisms. The necessity of the virus envelope for attachment and subsequent infection has been questioned, but attachment and penetration appears to be more efficient when the virus is enveloped (20).

The exact serological differences of HSV-type 1 and HSV-type 2 appear to reside in the capsid of the virion (21). These differences in protein makeup could lead to a preferential interaction of DMBA with one and not the other. The specificity of interaction of carcinogenic aromatic hydrocarbons with specific tissue proteins has been shown by Heidelberger and Moldenhauer (16). A specific reaction of DMBA with HSV-type 2 capsid protein could again lead to inactivation or charge alteration and affect viral adsorption, penetration, or uncoating.

The interaction of DMBA with DNA has been extensively studied by several investigators who have found that the reaction of DMBA with DNA is of a low magnitude and appears to be stable and irreversible (17, 18). The DNA of HSV-types 1 and 2 have been shown to differ slightly in density and in guanine plus cytosine content (22, 23). The interaction of DMBA with DNA of HSV could lead to a mutagenic effect of HSV so that it is unable to successfully complete a productive infection. The direct mutagenic effect of DMBA has been shown with bacterial viruses (24), but to our knowledge not with animal viruses. Whether or not the reaction of this carcinogen with a potentially oncogenic virus has a direct effect on the ultimate neoplastic potential of the virus remains to be determined.

Summary. A carcinogenic polycyclic aromatic hydrocarbon, DMBA, was found to inactivate the infectivity of herpes simplex virus type 2 to a greater degree than the infectivity of herpes simplex virus type 1. The reaction was concentration and temperature dependent. The noncarcinogenic polycyclic aromatic hydrocarbon, anthracene, did not affect the infectivity of either type of herpes simplex virus.

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