Inhibitory Effect of Carcinogenic Aromatic Hydrocarbons on Transformation of 3T3 Cells by SV 40[1] (36591)

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The demonstration of an inhibitory effect of 7,12-dimethylbenz[*a*]anthracene (DMBA) and 3-methylcholanthrene (3-MC) on Friend and Rauscher virus leukemias (1–4) led to investigation of the effect of these carcinogens on other virus-induced neoplasms. Because of the difficulty in measuring objectively the inhibition of solid neoplasms *in vivo*, the results of the present study were based on the effect of the two carcinogens on the *in vitro* transformation of 3T3 cells by SV 40 virus.

Materials and Methods. Cell lines and virus. The mouse fibroblast line 3T3 (5–7) was grown and maintained in Dulbecco and Vogt's modification of Eagle's basal medium, supplemented with 10% fetal calf serum, 25 units/ml of penicillin, and 25 μg/ml of streptomycin (hereafter referred to as "growth medium"). The 3T3 cells were maintained as described by Todaro and Green (6) to preserve high contact sensitivity.

SV 40 virus (strain 776) was grown and titered in the African green monkey cell line BSC-1 (8) using medium 199 with Earle's balanced salt solution containing 20% fetal calf serum, 0.1% yeastolate, 50 units/ml of penicillin, and 50 μg/ml of streptomycin. The virus pool used had a titer of 10^9 $TCID_{50}$/ml, determined by the method of Reed and Muench (9). The identity of the virus was confirmed by production of neoplasms after subcutaneous inoculation of newborn Syrian hamsters, by neutralization tests using calf SV 40 antiserum (Baltimore Biological Laboratory), and by demonstration of the SV 40 T antigen in the nuclei of the BSC-1 cells.

Chemical carcinogens. 1. Effect on plating efficiency of 3T3 cells. Stock solutions of 4 mg of DMBA and 3-MC (Eastman Organic Chemicals) in 5 ml of dimethylsulfoxide (DMSO) were stored in amber-colored bottles at room temperature and diluted in growth medium at the time of use. To avoid toxic concentrations of the carcinogens, their effect on the plating efficiency of 3T3 cells was determined as follows. Stock 3T3 cells were dissociated with 0.25% trypsin and viable cell counts were performed in a hemocytometer using the dye exclusion technique with 0.5% trypan blue. The cell suspension was diluted so that approximately 180 viable cells were contained in 0.1 ml which was placed into each of 60 plastic tissue culture dishes. To each of a series of 6 dishes, 0.5 ml of one of four dilutions of carcinogen (2.0, 0.2, 0.02, and 0.002 μg/0.5 ml) was added. Controls consisted of 6 dishes with untreated cells and 6 dishes with cells plus 0.5 ml of a 1:100 dilution of DMSO. All dishes received an additional 3.5 ml of growth medium and were incubated at 37° in a humid atmosphere containing 5% CO_2. The medium was replaced in all dishes on day 7. On day 14, the clones were fixed for 30 min with 10% formaldehyde in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) and stained for 2 min with crystal violet. The plating efficiency was determined according to the formula:

$$\text{Plating efficiency} = \frac{\text{clones formed}}{\text{cells plated}} \times 100.$$

2. Effect of transformation of 3T3 cells by SV 40 virus. Three separate experiments were

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TABLE I. Effect of DMBA and 3-MC on Plating Efficiency of 3T3 Cells.

Treatment (μg/ml)		No. of clones in 6 plates	Plating efficiency (%)	
			Absolute[a]	Relative[b]
None		263	24.4	100.0
DMSO		259	24.0	98.8
DMBA	0.5	3	0.3	1.2
	0.05	22	2.1	8.6
	0.005	95	8.8	36.1
	0.0005	242	22.4	92.0
3-MC	0.5	199	18.2	74.8
	0.05	252	23.3	96.0
	0.005	240	22.2	91.0
	0.0005	264	24.4	100.0

[a] Percentage of cells forming a clone.

[b] Percentage of cells forming a clone relative to the untreated controls.

carried out which differed only in the time at which the carcinogens were added to the cell cultures.

For each experiment 3 to 5 $\times$ 10^4 3T3 cells were plated in each of 6 tissue culture dishes containing 4 ml of growth medium. Two of the cultures subsequently were treated with DMBA (0.0005 μg/ml), two with 3-MC (0.05 μg/ml) and the remaining two served as controls. The cell cultures were incubated for 48 hr at 37° in a humid atmosphere containing 5% CO_2. The medium was then removed, and 0.5 ml of the virus pool was added to one designated DMBA, one designated 3-MC, and one control plate. The 3 other plates received 0.5 ml of growth medium. All dishes were incubated for 3 hr and rotated every 15–20 min. After virus adsorption, the plates were rinsed twice with growth medium; 4 ml of growth medium was added to each plate and incubation was continued for 24 hr. The cells were then dissociated with 0.25% trypsin and a viable cell count was done. The cells were diluted so that 150–300 viable cells from each of the original 6 plates were placed into each of 6–10 dishes with 4 ml of growth medium and incubated for 14 days. The medium was changed on day 7.

In Expt. 1 the carcinogens were added at the time of the original plating of the cells and, consequently, were in contact with those cells for 48 hr before their exposure to the virus. In Expt. 2 the chemicals were added simultaneously with the virus and were in contact with the cells for 3 hr. In Expt. 3 the chemicals were added 24 hr after virus infection and were in contact with the cells for 7 days.

Transformation efficiency was determined on day 14. Since the "background" of spontaneously transformed clones interfered with the analysis of the results, calculation of transformation efficiency was based on the presence of SV 40 T antigen in transformed clones. For this purpose, all plates were rinsed twice in CMF-PBS, fixed in absolute ethanol for 10 min at —60°, and air-dried at 4°. The plates were examined microscopically, the clones were counted, and their location was marked. One drop of a 1:5 dilution of hamster anti-T serum, prepared from SV 40 tumor bearing hamsters, was placed on each clone. The plates were incubated at 37° for 30 min and rinsed three times with CMF-PBS. Fluorescein-labeled rabbit anti-hamster serum (Microbiological Associates) was layered over each clone, the plates reincubated for 30 min, and rinsed twice with CMF-PBS. Coverslips were mounted over the clones with buffered (pH 7.4) glycerol. Microscopic examination using a darkfield condenser and an ultraviolet light source showed fluorescing nuclei in the positive cells.

3. Direct effect of carcinogens on infectivity of SV 40 virus for BSC-1 cells. Tenfold dilutions (200 to 0.002 μg/0.1 ml) of the carcinogens were prepared in medium 199 and 0.1 ml of each dilution was mixed with 0.2 ml of virus suspension. These mixtures were incubated at 37° for 30 min and logarithmic dilutions were made with medium 199. One-tenth milliliter amounts of the dilutions 10^{-3} to 10^{-7} were placed on triplicate monolayer tube cultures of BSC-1 cells, and were permitted to absorb for 60 min at 37°. Then 1 ml of medium 199 with Earle's balanced salt solution containing 2% fetal calf serum, 50 units/ml of penicillin, and 50 μg/ml of streptomycin were added, and the tubes were incubated at 37° for 14 days. All

TABLE II. Effect of DMBA and 3-MC Added (A) 48 hr Before (48 hr Cell Contact), (B) Together with (3 hr Cell Contact), and (C) 24 hr After (7 Days Cell Contact) SV 40 on the Transformation Efficiency of 3T3 Cells as Measured by Presence of SV 40 T Antigen in the Transformed Clones.

Treatment	No. of transformed clones (%)		
	(A)	(B)	(C)
None	0	0	0
DMBA	0	0	0
3-MC	0	0	0
SV 40	3.8	2.8	4.5
SV 40 + DMBA	3.1	3.0	2.0
SV 40 + 3-MC	3.2	1.8	1.0

tubes were examined daily, scored for CPE and the $TCID_{50}$/ml for each treatment was determined according to the method of Reed and Muench (9).

Results. The toxic effect of DMBA and 3-MC on the plating efficiency of 3T3 cells is presented in Table I. Based on the results of these experiments, the two carcinogens were subsequently used in concentrations which had only a mild effect on the plating efficiency, *i.e.*, 0.0005 μg/ml of DMBA and 0.05 μg/ml of 3-MC.

The effect of DMBA and 3-MC on the transformation of 3T3 cells by SV 40 is presented in Table II. When the carcinogens were in contact with the 3T3 cells for 48 hr before the medium was changed and the virus inoculated (A group) or for 3 hr after virus inoculation and before the medium was changed (B group) no significant effect on the number of transformed clones was observed. Conversely, contact of the carcinogens with the cells for 7 days (C group), beginning 24 hr after virus inoculation, reduced the number of transformed clones to a degree which was statistically significant as determined by analysis of variance with a factorial design.[3]

Finally, neither DMBA nor 3-MC in concentrations from 0.002 to 200 μg/ml had any direct effect on the infectivity titers of SV 40 virus for BSC-1 cells.

Discussion. The depressive effect of carcinogenic polycyclic aromatic hydrocarbons (CPAH) on transformation by a DNA virus *in vitro*, as described in this study, may operate in a manner analogous to the previously observed phenomenon (1, 2) of delay in malignant transformation *in vivo* by such carcinogens in the RNA virus-induced leukemias of Friend and Rauscher.

Among the agents having an effect on viruses, interferon has attracted the greatest attention. Its influence on productive infection by many DNA and RNA viruses is well known, but its effect on transformation by some oncogenic viruses has also been reported. Exogenous interferon has been shown to inhibit transformation of 3T3 cells by SV 40 virus (10). On the other hand, CPAH have been reported to depress interferon production, thereby enhancing viral replication (11). However, even if CPAH stimulated instead of depressed production of interferon under certain conditions, this agent could be excluded as the cause of the depressive effect in our experiment since 3T3 cells have been shown to be resistant to the effect of interferon 24 hr after infection with SV 40 (12).

Also, CPAH have been reported to exert a direct suppressive effect on replication of DNA viruses, but not on RNA viruses (13). But this observation does not explain our results because the concentration of DMBA in their experiments was much greater (0.01 to 10 μg/ml) than in our experiments and, therefore, in the toxic range. Also, we were unable to show a suppression of SV 40 virus replication in the BSC-1 cell line by CPAH. Finally, the 3T3 cell line reacts to SV 40 only by transformation and not by productive infection.

Since an influence on the virus itself does not seem likely, our results might be explained best by a direct action of the CPAH on synthesis of cellular DNA, probably by binding of the carcinogens to DNA. It has been shown that 0.1 μg/ml of DMBA inhibited cellular DNA synthesis in normal mouse and hamster embryo cell cultures within 2 hr, reduced the mitotic activity by

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50% at 4 hr, and produced a maximal level of binding of ^{3}H-labeled DMBA to DNA after 24 hr (14, 15). The lack of effect when the 3T3 cells were exposed to the CPAH for 48 and 3 hr might be due to the small amount of DMBA used in our experiments (0.0005 μg/ml), such a concentration being effective only during the longer exposure time of 7 days. Length of contact and speed of absorption might play a role, as also might a greater sensitivity of the cells to CPAH after the virus has turned on cellular DNA synthesis leading to the phenomenon of transformation. This assumption derives support from experiments in which polyoma-transformed 3T3 cells have been shown to be more sensitive to 8 and 11 day exposure to the toxic doses of 0.01 and 0.1 μg/ml of DMBA than untransformed 3T3 cells (16). Furthermore, the sevenfold increase in the number of transformed clones of 3T3 cells exposed for 2 weeks to a tumor-promoting phorbol ester of croton resin was only threefold when this exposure followed treatment with 0.1 μg/ml of DMBA for 2 weeks (17). The transforming effect of the phorbol ester might be analogous to the transforming effect of SV 40 in our experiments.

Other substances which interfere with DNA synthesis might be expected to have a similar effect as the CPAH. However, the thymidine analogs, 5-iodo-2′-deoxyuridine (IUDR) and 5-bromo-2′-deoxyuridine (BUDR), which are incorporated into the DNA of mammalian cell cultures during replication in place of thymidine have been shown to increase the number of SV 40-transformed clones of 3T3 cells, as long as these compounds were added to growing DNA-synthesizing cultures either before or after infection (18). The difference might be due to the fact that in those experiments the thymidine analogs produced a marked decrease in plating efficiency and increased the number of transformed clones only in the surviving cells, whereas in our experiments the CPAH did not affect the plating efficiency, *i.e.*, did not have the toxic effect of the thymidine analogs.

Summary. The carcinogenic polycyclic aromatic hydrocarbons 7,12-dimethylbenz[*a*]anthracene (0.0005 μg/ml) and 3-methylcholanthrene (0.005 μg/ml) did not have a toxic effect on the plating efficiency of 3T3 cells. The number of SV 40-transformed clones of 3T3 cells was decreased when these carcinogens were added 24 hr after virus inoculation and were in contact with the cells for 7 days. However, there was no effect when the carcinogens were in contact with the cells for 48 hr before or for 3 hr immediately after virus inoculation. No direct effect of the carcinogens on the infectivity of SV 40 was observed when the virus was incubated with from 0.002 to 200 μg/ml of the carcinogens and subsequently titered in the BSC-1 cell line. These findings confirm *in vitro* for another oncogenic virus the previously described inhibitory effect of these carcinogens on Friend and Rauscher virus leukemias. The most likely explanation seems to be an effect of the carcinogens on the synthesis of cellular DNA during the period when the virus directs the events leading to transformation.

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