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Transformation of Hamster Cells *In Vitro* by Polycyclic Hydrocarbons without Cytotoxicity

(cloning efficiency/carcinogen/protective agents/frequency of transformation)

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ABSTRACT **Treatment with either a flavone or benz(α)anthracene before the addition of a potent carcinogen, benzo(α)pyrene or 3-methylcholanthrene, enhanced the transformation of Syrian hamster cells that are seeded in order to form colonies. 7,8-benzoflavone and benz(α)-anthracene prevented the cytotoxicity by the carcinogens, while 5,6-benzoflavone did not. The results clearly indicate that it is possible to dissociate the transforming from the toxic metabolic properties of benzo(α)pyrene and 3-methylcholanthrene.**

The addition of carcinogenic polycyclic hydrocarbons to hamster secondary cultures *in vitro* results in transformation of the hamster cells, and is accompanied by toxicity that is related to the dose of hydrocarbon added (1–3). Tritiated 7,12-dimethylbenz(α)anthracene (Me_2BzA) has been used to demonstrate that within 6 hr, the percentage of labeled cells ranges from 65–98, while the amount of label incorporated per nucleus continues to increase with the length of exposure (4). This nonspecific labeling is evidence that the carcinogen can enter almost all cells of such a mixed population. Utilization of quantitative tissue culture techniques has made possible the demonstration that the proportion of cells capable of forming transformed colonies represents only a small number of those that actually are able to take up the carcinogen. It has been concluded that a large proportion of the cells is either normally sensitive to the cytotoxic effect of the carcinogen or resistant to the cytotoxic effect and incapable of being transformed, while an extremely small proportion can be transformed (5,6).

Results of more recent studies indicate that mass cultures may be protected from the cytotoxic effects of polycyclic hydrocarbon carcinogens by inhibition of the induction of aryl hydrocarbon(benzo[α]pyrene) hydroxylase. Accompanying such treatment was the prevention of inhibition of cell multiplication that is normally induced by these carcinogenic hydrocarbons in hamster-embryo cell cultures (7). The question remained whether the reduction or prevention of cytotoxicity produced by known carcinogens would be accompanied by a similar reduction in the percentage of colonies transformed. Failure to find decreased efficiency of transformation would be consistent with the concept that cytotoxicity and transformation are independent phenomena and do represent two different responses of a cell population to the same insult. In the current study, 7,8-benzoflavone and benz(α)-anthracene produced no toxicity, as indicated by the effect on cloning efficiency, while 5,6-benzoflavone was toxic. Only benz(α)anthracene was a weak transforming agent (3); no transformed colonies were found with either naphthoflavone. When potent carcinogens were added, transformation occurred in the presence of all three compounds and was generally enhanced, while the toxicity generally produced by carcinogenic polycyclics was prevented by 7,8-benzoflavone and benz(α)anthracene.

Abbreviations: Me_2BzA, 7,12-dimethylbenz(α)anthracene; BzA, benz(α)anthracene; MCA, 3-methylcholanthrene; BzP, benzo(α)-pyrene; 7,8-BzFl, 7,8-benzoflavone (α-naphthoflavone); 5,6-BzFl, 5,6-benzoflavone(β-naphthoflavone).

MATERIALS AND METHODS

Chemicals. 5,6-benzoflavone [β-naphthoflavone (5,6-BzFl)] was purchased from K & K Laboratories; 7,8-benzoflavone [α-naphthoflavone (7,8-BzFl)], benz(α)anthracene (BzA), benzo(α)pyrene (BzP), and 3-methylcholanthrene (MCA) were purchased from Eastman Kodak Co., and acetone (analytical grade) was purchased from Mallinckrodt Co. Chemicals were dissolved in acetone and added to warm complete medium to make stock solutions.

Transformation Assay. The transformation assay has been described in detail (3, 5). Pregnant Syrian hamsters were obtained from Mr. S. Poiley, National Cancer Institute. Primary and secondary cultures from whole embryos were prepared and grown in Dulbecco's Modification of Eagle's Medium, supplemented with 10% fetal bovine serum. 500 2-day cultured cells from secondary cultures of hamster embryo cells were plated in 2 ml of medium in 60-mm plastic Petri dishes (Falcon), containing a 24-hr culture of feeder layer of 6×10^4 irradiated hamster cells in 2 ml of complete Dulbecco's medium. 24 hr later, concentrated solutions of naphthoflavone or BzA in 2 ml of medium were added to the cultures. The next day (48 hr after the nonirradiated hamster cells had been plated) the carcinogen, in diluted stock solution, was added to 2 ml of medium so that the final concentration was that indicated in the Tables; the final concentration of the protective agents varied, since they were either added once, as indicated previously, or given in multiple treatments by addition of the protective agent in 0.1 ml of medium two more times at 48-hr intervals. The medium was not changed during the course of the experiment. The dishes were kept in a humidified 10% CO_2–air environment in an incubator at 37°C. 8 days after hamster cells had been seeded, the colonies were fixed in methanol and stained with Giemsa.

A stereoscopic microscope was used at a relatively low magnification to count colonies of a minimum of 2-mm diameter. For the determination of cloning efficiency, the average number of colonies per plate was divided by the number of cells seeded per plate multiplied by 100. The frequency of transformation was calculated on a per cell and

TABLE 1. *Transformation of hamster embryo cells treated with benzo(α)pyrene or 3-methylcholanthrene: increased cloning efficiency and enhanced transformation with 7,8-benzoflavone*

7,8-Benzo-flavone	Carcinogen (μg/ml of medium)	Total no. of colonies	Cloning efficiency (%)	No. of transformed colonies	Transformed colonies per cell × 10^3	Enhance-ment†	Transformed colonies / Total no. of colonies (%)	Enhance-ment†
0	0	410	13.7	0	0	—	0	—
5*	0	369	12.3	0	0	—	0	—
	BzP							
0	1	1016	8.46	25	2.08	—	2.46	—
0	5	394	7.17	21	3.80	—	5.33	—
1	1	754	12.6	69	11.50	3.53	9.15	3.72
5	1	612	13.6	80	17.78	5.45	13.07	5.31
1 × 3‡	1	713	13.0	51	9.27	2.85	7.16	2.91
1	5	706	12.8	62	11.27	2.97	8.78	1.65
5	5	717	13.0	55	10.00	2.63	7.67	1.43
1 × 3	5	714	13.0	38	6.91	1.82	5.33	1.00
	MCA							
0	2.5	664	9.6	20	3.43	—	3.46	—
1	2.5	768	12.8	42	7.00	2.04	5.47	1.58
1 × 3	2.5	777	12.9	57	9.50	2.77	7.31	2.11

* Concentration (μg/ml of medium).

† Frequency of transformed colonies per cell or of total colonies from double treatment, as compared to frequency of colonies treated with carcinogen only; results from cultures treated with carcinogen only are defined as 1.0.

‡ Three treatments on alternate days.

total colony count basis. Clonal morphology, both normal and transformed (indicated by a random crisscross pattern of spindle cells not seen in control colonies), was determined at ×40 magnification.

RESULTS

When several concentrations of 7,8-BzFl, 5,6-BzFl, or BzA were added to hamster embryo cells, the cloning efficiency was unaffected except by 5,6-BzFl at a concentration of 5 μg/ml of medium. With 5,6-BzFl, the reduction in cloning efficiency was greater than that which was found with the carcinogen alone and equal to that which was obtained when 5 μg of BBzFl was used in conjunction with 1 μg of BzP. As previously demonstrated, treatment with BzA at the highest concentration used (10 μg/ml of medium) produced a 0.5–1.0% transformation (3). No transformed colonies were observed in any of the control Petri dishes. The results reported in Tables 1–3 for the potent carcinogens, BzP or MCA, and the dose–response relationships are consistent with those that we have published (3, 5). The series of experiments reported in the tables have been repeated five times during the past year; the results were consistent, so only one set of data is presented. Each value in the results was determined from analyses of a minimum of 12 dishes.

Treatment of cells with 7,8-BzFl before the addition of either BzP or MCA and with either low, high, or multiple doses of the "protective agent" (see *methods*) was sufficient to raise the cloning efficiency above that obtained with the carcinogen only, to a frequency that approached that of solvent-treated cells (Table 1). Enhancement on either a per cell or a per colony basis, relative to controls, was greatest when treatment with 5 μg of 7,8-BzFl was combined with post-treatment with 1 μg of BzP/ml of medium. Other combinations of 7,8-BzFl and BzP produced various degrees of enhancement, but in no case was there a reduction in the transformation rate relative to treatment with the carcinogen only. The data obtained with MCA prove again that pretreatment with 7,8-BzFl protects from the toxicity ordinarily seen with MCA and increases the frequency of transformation. The transformation increases on either a per cell or colony basis and the enhancement factors are consequently also increased.

When 5,6-BzFl is substituted for 7,8-BzFl, in combination with BzP or MCA (Table 2), similar results are seen. The frequencies of transformation on either a per cell or colony basis in cultures pretreated with 5,6-BzFl exceed those found in cultures treated with carcinogren only in all instances, with the exception of cultures pretreated with 5 μg of 5,6-BzFl/ml of medium and then with 5 μg of BzP/ml medium. A single treatment with 5 μg of 5,6-BzFl, followed by 1 μg of BzP, produced maximum enhancement and, on a colony basis, a 5-fold increase in transformation. The addition of 5 μg of 5,6-BzFl followed by 5 μg of BzP resulted in an enhancement factor of less than 1 (0.86) on a cell basis, while on a colony basis, the enhancement factor was 1.41. Although a nontransforming agent, 5,6-BzFl by itself is capable of decreasing the cloning efficiency at concentrations of 1 or 5 μg/ml of medium. The combination experiments performed with MCA are similar to those obtained with 7,8-BzFl with a single treatment at a concentration of 1 μg/ml, but are lower with 5,6-BzFl when multiple treatments are used at a concentration of 1 μg/ml of medium, as compared to multiple treatment with 1 μg of 7,8-BzFl and then with MCA. In this last case, even the transformation per cell or transformation per colony is reduced in cultures pretreated with 5,6-BzFl.

The results of the substitution of BzA for one of the naphthoflavones are shown in Table 3. Protection from toxicity is seen again, since either single or multiple treatment with

TABLE 2. *Transformation of hamster embryo cells treated with benzo(α)pyrene or 3-methylcholanthrene: increased cloning efficiency and enhanced transformation with 5,6-benzoflavone*

5,6-Benzo-flavone	Carcinogen (μg/ml of medium)	Total no. of colonies	Cloning efficiency (%)	No. of transformed colonies	Transformed colonies per cell × 10³	Enhance-ment†	Transformed colonies / Total no. of colonies (%)	Enhance-ment†
0	0	410	13.7	0	0	—	0	—
5*	0	342	5.7	0	0	—	0	—
	BzP							
0	1	1016	8.46	25	2.08	—	2.46	—
0	5	394	7.17	21	3.80	—	5.33	—
1	1	582	9.7	38	6.33	1.95	6.53	2.65
5	1	300	5.5	39	7.09	2.18	13.00	5.28
1 × 3‡	1	671	11.2	36	6.00	1.84	5.37	2.18
1	5	443	9.8	28	6.22	1.64	6.32	1.19
5	5	240	4.4	18	3.27	0.86	7.50	1.41
1 × 3	5	377	10.8	33	9.43	2.48	8.75	1.64
	MCA							
0	2.5	664	9.6	20	3.43	—	3.46	—
1	2.5	728	12.1	42	7.00	2.04	5.77	1.66
1 × 3	2.5	635	12.7	36	7.20	2.09	5.67	1.63

* Concentration (μg/ml of medium).
† Frequency of transformed colonies per cell or of total colonies from double treatment, as compared to frequency of colonies treated with carcinogen only; results from cultures treated with carcinogen only are defined as 1.0.
‡ Three treatments on alternate days.

BzA resulted in a higher cloning efficiency than that obtained in cultures treated with the carcinogen alone. At the same time, the transformation rate is higher than that obtained with the carcinogen alone. BzA only did give rise to a few

TABLE 3. *Transformation of hamster embryo cells treated with benzo(α)pyrene or 3-methylcholanthrene: increased cloning efficiency and enhanced transformation with benz(α)anthracene*

Benz(α)-anthracene	Carcinogen (μg/ml of medium)	Total no. of colonies	Cloning efficiency (%)	No. of transformed colonies	Transformed colonies per cell × 10³	Enhance-ment†	Transformed colonies / Total no. of colonies (%)	Enhance-ment†
0	0	410	13.7	0	0	—	0	—
10*	0	786	13.1	4	0.66	—	0.51	—
	BzP							
0	1	1016	8.46	25	2.08	—	2.46	—
0	5	394	7.17	21	3.80	—	5.33	—
1	1	638	11.6	32	5.82	1.79	5.02	2.04
5	1	805	13.4	87	14.5	4.45	10.81	4.39
1 × 3‡	1	675	12.3	58	10.54	3.23	8.59	3.49
1	5	638	11.6	38	6.91	1.82	5.96	1.12
5	5	642	12.8	50	10.00	2.63	7.79	1.46
1 × 3	5	737	12.3	57	9.50	2.50	7.73	1.45
10	5	576	12.8	133	32.3	8.50	23.07	4.33
10 × 3	5	812	13.5	118	21.3	5.61	14.50	2.72
	MCA							
0	2.5	664	9.6	20	3.43	—	3.46	—
1	2.5	640	10.7	28	4.67	1.36	4.38	1.26
1 × 3	2.5	728	12.1	57	9.50	2.77	7.83	2.23
10	2.5	747	13.6	80	20.3	5.91	10.69	3.09
10 × 3	2.5	749	13.6	57	10.4	3.03	7.61	2.18

* Concentration (μg/ml of medium).
† Frequency of transformed colonies per cell or of total colonies from double treatment, as compared to frequency of colonies treated with carcinogen only; results from cultures treated with carcinogen only are defined as 1.0.
‡ Three treatments on alternate days.

transformed colonies at the highest concentration used (10 μg/ml medium). Similar maximal enhancements of transformation were noted with single treatment of cultures with 5 or 10 μg of BzA, followed by 1 or 5 μg of BzP on a per cell or per colony basis; over all, enhancement on a cell basis was higher with BzA and BzP than with any other combination of protective agent and carcinogen. Enhancement of transformation with MCA was maximum with a single treatment of cultures with 10 μg of BzA on both a cell and colony basis, and exceeded the results obtained with all other protective agents. Transformation with BzA and MCA was again increased without any reduction in cloning efficiency. In fact, with pretreatment with 10 μg of BzA and then with 1 or 5 μg BzP, or with 2.5 μg of MCA per ml of medium, the cloning efficiency was equal to that obtained with untreated controls.

DISCUSSION

In experiments with hamster embryo cells that are plated to form colonies, prior treatment with 7,8-BzFl or BzA prevented the toxicity ordinarily associated with the potent carcinogens BzP or MCA; results with 5,6-BzFl were more variable, since it was capable of inducing some toxicity. Transformation rate increased on both a cell and colony basis. Thus, these results present clear evidence that the toxic and transforming activity of carcinogenic polycyclic hydrocarbons are dissociable and that the two processes, as has been suggested, are different (5, 6, 8). These results do not negate previous reports of the relationship of cytotoxicity to concentration and potency of the carcinogen. Some transformed colonies may be sensitive to higher concentrations of the same carcinogen; thus ordinarily these colonies may be eliminated, while additional colonies become transformed.

The presence of the protective agent during exposure to the carcinogen seems to increase the rate of metabolic conversions or formation of a theoretical carcinogenic polycyclic compound to its reactive carcinogenic form.

In vitro, 7,8-BzFl has been demonstrated to protect hamster embryo cells from the inhibition of cell multiplication caused by BzP or Me_2BzA. Experiments with mouse skin demonstrated that 5,6-BzFl also shows biological affinity for the same enzyme system and the capacity for some inducing activity (9).

Experiments with BzA demonstrated that a high induced plateau activity of aryl hydroxylase is observed 24 hr after the addition of the compound to monolayers of hamster embryo cells (9). Because we were interested in determining whether or not transformation occurred at the time of maximum protection, we added BzA or either naphthoflavone 24 hr before the addition of the potent carcinogen, BzP or MCA.

A number of studies with animals have demonstrated protection against the neoplastic effects of chemical carcinogens by the addition of other carcinogens. Polycyclic aromatic hydrocarbons that are weakly carcinogenic have been shown to prolong the mean latent period of tumor production and reduce the mean yield of tumors induced in the skin of mice by potent carcinogens, such as MCA or Me_2BzA (11, 12). Exceptions do exist. One weak carcinogen, BzA, when used as an inhibitor of Me_2BzA-induced skin carcinogenesis, prolonged the mean latent period of tumor production but did not reduce the mean tumor yield (11). More recently, it has been reported that induction of changes in microsomal enzyme activities can cause protection against the carcinogenic effect of polycyclic hydrocarbon carcinogens. Two experimental models have been used mainly for this purpose: the formation of mammary tumors in rats given oral doses of Me_2BzA, and the determination of skin tumor formation in mouse skin. Induction of increased polycyclic-hydrocarbon hydroxylase activity by several different flavones and other compounds before the administration of Me_2BzA inhibits breast tumor formation* (13); SKF525-A, an inhibitor of microsomal enzyme activity, leads to an increased incidence of tumor formation (14). The formation of skin tumors in mice has been inhibited by pretreatment of the skin with 5,6-BzFl as an inducing agent and BzP as a carcinogen (15); pretreatment with 7,8-BzFl, an inhibitor of the enzyme or a weakly inducing compound has been responsible for the inhibition of tumors when given 5 min before treatment with Me_2BzA (9).

Current evidence indicates that the carcinogenic polycyclic hydrocarbons are probably converted to active derivatives (16) and that microsomal enzyme systems are required to enhance the formation of BzP metabolites that bind to DNA *in vitro* (17, 18). The requirement for metabolic activation is further confirmed by the evidence that epoxides or diols of weakly carcinogenic compounds such as benz(α)anthracene are more active than the parental compounds themselves in producing transformation (19). Our positive results and those obtained with K-region epoxides support the concept that metabolically activated intermediates are required for transformation. Compounds that have been reported to increase the enzyme activity enhance the transformation rate that is obtained with BzP or MCA.

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