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Induction of Mutagenesis and Alterations in Gene Expression by Tumorigenic Chemicals.

by

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Both epidemiological retrospective surveys and studies with experimental animals provide evidence that some chemicals in our environment are responsible for a significant proportion of human cancers (Higginson, 1972).

In view of the complexity in studying chemical carcinogenesis in whole animals, it is useful to use simple mammalian cell cultures for such studies. Cell culture systems may also be useful for the identification of potentially hazardous chemicals, since one must presume that there are many environmental chemicals whose potential carcinogenic hazard has not yet been established.

Malignant transformation of cells in culture by chemical carcinogens has been achieved in normal cells and permanent cell lines (Berwald and Sachs, 1965; Heidelberger, 1973). Using these in vitro systems, it was also established that transformed cells are induced rather than selected by the carcinogens (Huberman and Sachs, 1966, 1968; Mondal and Heidelberger, 1970). These results and the observations that carcinogens bind to cellular macromolecules, including DNA in transformable cells (Brookes and Lawley, 1967; Kuroki and Heidelberger, 1971; Huberman and Sachs, 1977), suggest that the heritable phenotypic changes obtained during chemically induced in vitro cell transformation may result from somatic cell mutations. Based on these and other studies, it was possible, by determining mutagenic activity, to devise simple in vitro systems for the identification of potential chemical carcinogens. Recently, Ames and his associates have developed a simple assay for testing mutagenicity by chemical carcinogens using their Salmonella typhimurium as target organisms for mutagenesis and liver homogenates for carcinogen activation. Based on a consistent finding that the majority of the tested carcinogens were mutagenic, Ames has proposed that the assay be used as a screen for potentially hazardous compounds (Ames, et al., 1973;

McCann, et al., 1975). However, liver subcellular fractions readily activate a wide spectrum of compounds into potent mutagens including those which are not necessarily carcinogenic for the liver. In addition, subcellular preparations differ from intact cells in the profile of the metabolites and DNA adducts formed after metabolism of various potent carcinogens such as aflatoxin B_1, benzo(a)pyrene and 7,12-dimethylbenz(a)anthracene (Newbold, et al., 1977; Selkirk, 1977; Decad, et al., 1977; Bigger, et al., 1978). To circumvent these difficulties, we have devised a system of mammalian cell-mediated mutagenesis (Huberman, 1975, 1976, 1977; Huberman and Sachs, 1974, 1976; Langenbach, et al., 1978a, 1978b). In this assay, Chinese hamster V79 or Chinese hamster ovary (CHO) cells, which do not exhibit mixed function oxidase activities and therefore do not activate many chemical carcinogens, are cocultivated with lethally irradiated cells that contain the enzymes required for metabolic activation.

During cocultivation, the reactive metabolites are apparently transferred from the metabolizing cell to the cells used for testing mutagenesis, where they induce mutation. Using this cell-mediated mutagenesis system, it was possible to demonstrate a relationship between carcinogenicity and mutagenicity by polycyclic hydrocarbons, nitrosamines and aflatoxins (Huberman and Sachs, 1976; Langenbach, et al., 1978a), and to show that metabolic activation of carcinogens to mutagens is cell specific (Langenbach, et al., 1978b).

Cell mediated mutagenesis by chemical carcinogens

To determine the relationship between mutagenesis and carcinogenesis, a series of 11 polycyclic hydrocarbons with different degrees of carcinogenicity

were tested in the cell-mediated mutagenesis assay for the induction of ouabain-resistant mutants (Table 1). After cocultivation of the Chinese hamster V79 cells with golden hamster embryonic cells, which are capable of activating polycyclic aromatic hydrocarbons, four carcinogenic hydrocarbons [7,12-dimethylbenz(a)anthracene, benzo(a)pyrene (BP), 3-methylcholanthrene, and 7-methylbenz(a)anthracene] induced ouabain-resistant mutants, whereas five noncarcinogenic hydrocarbons [benzo(e)pyrene, benz(a)anthracene, phenanthrene, pyrene, and chrysene] were not mutagenic (Table 1). Dibenz(a,c)anthracene and dibenz(a,h)anthracene, which have been reported as noncarcinogenic in golden hamsters (IARC, 1973; National Institutes of Health, 1973), showed a weak mutagenic effect. In the presence of aminophylline, which enhances polycyclic hydrocarbon metabolism (Huberman and Sachs, 1976, 1977; Huberman et al., 1974; Yamasaki, 1975), there was a two- to fourfold increase in mutagenicity with BP and 3-methylcholanthrene. Dibenz(a,c)anthracene, which showed a low degree of mutagenicity without animophylline, showed a less than twofold increase of mutagenicity with aminophylline. Dibenz(a,h)anthracene, which was similar to dibenz(a,c)anthracene without aminophylline, showed a tenfold increase in mutagenicity with aminophylline (Table 2). These results indicate that there is a relationship between mutagenesis and the degree of carcinogenicity of polycyclic hydrocarbons after enhancement of their metabolism by aminophylline.

However, the above cultured golden hamster embryonic cells were incapable of activating many classes of chemical carcinogens, including most liver carcinogens. Therefore, to expand the uses of the cell-mediated mutagenesis system and allow study of liver carcinogens,

we developed a system for cocultivating primary liver cells and V79 cells. In this system, two classes of liver carcinogens, the nitrosamines (Table 3) and aflatoxins (Table 4), were metabolized by the liver cells to intermediates that were mutagenic to the V79 cells. The fact that the carcinogenic nitrosamines and the aflatoxins were mutagenic in the presence of liver cells but not in their absence suggests that the reactive intermediate(s) of these two classes of carcinogens are stable enough to be transferred from the liver cells to the V79 cells.

To determine whether a cell-type specificity in chemical carcinogenesis can be investigated by way of the cell mediated assay, we have cocultivated V79 cells with rat fibroblasts which are capable of metabolizing polycyclic aromatic hydrocarbons (fibroblast-mediated assay) and with rat liver cells which metabolize liver carcinogens (hepatocyte-mediated assay) in the presence of the carcinogens benzo(a)pyrene (BP) and aflatoxin B_1 (AF). Our results indicate mutagenic activity of BP in the fibroblast-mediated assay but not in the hepatocyte-mediated assay and the inverse situation with AF (Langenbach et al., 1978b). More specifically, BP in the fibroblast-mediated assay caused 17-, 34-, and 55-fold higher mutation frequencies than the control at 0.3, 1.0 and 3.0 $\mu g\ ml^{-1}$, respectively. While in the hepatocyte-mediated assay no significant increase was observed in the mutation frequencies at BP doses up to 3 $\mu g\ ml^{-1}$. Treatment of the V79 cells with AF at 0.3, 1.0 or 3.0 $\mu g\ ml^{-1}$ in the hepatocyte-mediated assay resulted in 10-, 20- and 40-fold higher mutation frequencies than the control. In the fibroblast-mediated assay, AF caused only a twofold increase at a dose as high as 3 $\mu g\ ml^{-1}$. These results indicate that by

employing appropriate cell culture systems, we can show that chemical carcinogens can be metabolized into mutagens for mammalian cells. In addition, both the degree of mutagenesis and cell-type specificity in such cell systems can be related to the carcinogenic potency and organ specificity of the tested chemicals.

Restoration of a "normal" functional state in cultured human leukemia and melanoma cells after treatment with tumor-promoting agents

Our previous studies and those of others are in agreement with the notion that somatic mutation may be the step which initiates carcinogenesis. However, our present state of knowledge indicates that cancer is a multi-stage process that may require a step(s) beyond the initial mutational event. Thus, it is possible that many environmental tumor-causing agents may act at the later stages of the carcinogenic process in a mechanism related to tumor promotion. A tumor promoter is an agent that is effective only when administered frequently after an initial treatment with a low dose of a carcinogen-mutagen and not when the sequence is reversed. Since certain initiating agents are presumably ubiquitous in the environment, the rate-limiting determinants in cancer causation in humans may be exposure to such tumor promoters.

Phorbol-12-myristate-13-acetate (PMA) and other related phorbol diesters, which are tumor promoters in the mouse, were found to inhibit cell differentiation in a series of murine cells in vitro (Cohen et al., 1977; Diamond et al., 1977; Ishii et al., 1978; Mufson et al., 1978; Yamasaki et al., 1977). These experiments suggest that tumor promoters may be gene modulators which can inhibit normal differentiation, thus allowing the

expression of the tumorigenic phenotype of the initiated cells. It was, therefore, of interest to determine whether such agents would affect cell differentiation in human cells in a manner similar to the mouse cells.

Our in vitro studies with some myeloid human leukemia and melanoma cells indicated that tumor-promoting agents, such as the phorbol diesters, induce rather than inhibit terminal differentiation (Huberman and Callaham, 1979; Huberman et al., 1979). Terminal differentiation in the human leukemic cells was expressed by an inhibition of cell growth and by the conversion of myeloblasts and promyelocytes, which are characteristic to leukemia cells, into myelocytes and metamyelocytes as well as banded and segmented cells characteristic of differentiating normal white blood cells. In the process of the conversion of the myeloid leukemic cells into mature myeloid cells, they acquire their normal differentiating properties, such as an ability to phagocytize microorganisms including yeast and to release lysozyme, an emzyme capable of lysing bacterial cell walls.

In the melanoma cells, as in the leukemic cells, these agents inhibit cell growth, stimulate melanin synthesis and induce the formation of dendrite-like structures characteristic of normal melanocytes. Terminal cell differentiation in the human leukemia and melanoma cells could be detected at concentrations of PMA as low as 10^{-11} to 10^{-10}M. Phorbol diester-like compounds may therefore offer a means by which some human tumors may be controlled by converting them into normally differentiating cells. However, studies on the susceptibility of a variety of different normal and malignant human tissues to the growth inhibitory effect of these types of compounds have to be performed before such an approach can be considered.

In the present experiments using human cells, tumor-promoting phorbol esters induced terminal differentiation while in other studies, in which avian and murine cells were employed, they inhibited differentiation. These results imply that human cells may respond differently from mouse and chicken cells to the biological effects of phorbol diesters. In view of this, extrapolation of data from mouse to human on the effect of chemicals, such as the tumor-promoting phorbol diesters, should be made with great caution.

TABLE 1. Induction of Ouabain-Resistant Mutants in the Cell-Mediated Assay by Different Carcinogenic Hydrocarbons[a]

Hydrocarbon	Concentration of hydrocarbon (μg/ml)	No. of ouabain-resistant mutants per 10^6 survivors
Control	0	1
Benzo(e)pyrene	1	1
Phenanthrene	1	1
Pyrene	1	1
Benz(a)anthracene	1	2
Chrysene	1	2
Dibenz(a,c)anthracene	1	3
Dibenz(a,h)anthracene	1	4
7-Methylbenz(a)anthracene	1	24
3-Methylcholanthrene	1	108
Benzo(a)pyrene	1	121
7,12-Dimethylbenz(a)anthracene	0.1	66

[a]The data are based on results from Huberman (1976) and from Huberman and Sachs (1976).

TABLE 2. Induction of Ouabain-Resistant Mutants in the Cell-Mediated Assay by Carcinogenic Polycyclic Hydrocarbons after Treatment with (+) or without (-) Aminophylline[a]

	No. of ouabain-resistant mutants per 10^6 survivors	
	(-)	(+)
Control	1	1
Pyrene	1	1
Phenanthrene	1	1
Dibenz(a,c)anthracene	3	5
Dibenz(a,h)anthracene	4	46
3-Methylcholanthrene	108	413
Benzo(a)pyrene	121	214

[a]Cells were treated with 1 μg/ml of the polycyclic hydrocarbons and 0.1 mM aminophylline. Data are based on results from Huberman and Sachs (1976).

TABLE 3. Induction of ouabain-resistant mutants by nitrosamines

Treatment*	Concentration (mM)	Cloning efficiency (%)	Ouabain-resistant mutants/10^6 survivors
Control		88	1
N-nitrosodimethylamine	4.5	18	115
	13.5	20	171
N-nitrosodiethylamine	0.5	94	2
	4.5	56	16
	13.5	44	30
	45.0	38	50
Nitrosomethyl-text-butylamine	0.5	88	1
	4.5	84	1
	13.5	85	1

*The number of V79 cells/T-flask was 0.6 X 10^6 when 10^7 liver cells were seeded. The cells were treated for 2 days with the chemicals, at which time they were trypsinized and reseeded to determine cloning efficiency and mutation frequency. At this time the V79 cell number/T-flask ranged from 1.2 X 10^6 to 3.3 X 10^6. Data are from Langenbach et al., 1978a.

TABLE 4. Induction of ouabain-resistant mutants by aflatoxins

Treatment*	Concentration (μM)	Cloning efficiency (%)	Ouabain-resistant mutants/10^6 survivors
Control		82	1
Aflatoxin B_1	0.2	64	7
	1.4	40	14
	3.2	26	19
	9.6	5	67
Aflatoxin G_2	0.2	89	1
	0.3	89	1
	1.4	79	1
	3.2	85	1

*See legend in Table 1.

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