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INDUCTION OF HYPERPLASIA AND ANAPLASIA BY CARCINOGENS IN ORGAN CULTURES OF MOUSE PROSTATE

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SUMMARY

In an effort to establish a test system to examine the carcinogenic potential of chemicals, mouse prostate explants were maintained as organ cultures and the effects of carcinogenic and noncarcinogenic compounds were examined at various intervals after treatment. The degree of hyperplasia produced by a compound was determined by the colcemid metaphase arrest technique. Extensive hyperplasia of the prostatic epithelium occurred at 8 days after treatment with 3-methylcholanthrene, the 11-12 epoxide of methylcholanthrene, benzo(a)pyrene and N-methyl-N-nitro-N-nitrosoguanidine. At 12 days most carcinogen-treated explants were anaplastic. The noncarcinogenic compounds, pyrene and phenanthrene, did not produce a mitotic stimulatory effect on the epithelium of the explants. The data suggest that the organ culture system of mouse prostate may be employed as a test system to obtain preliminary information regarding the carcinogenicity of a compound.

Key words: prostate; organ culture; carcinogens, hyperplasia.

INTRODUCTION

Cell and organ culture systems are important tools in studying a variety of biological problems and bypassing the in vivo complexities. Cell culture systems consisting primarily of fibroblasts have been used extensively for testing the carcinogenic potential of chemicals (1-12). However, the use of fibroblasts for the study of chemical carcinogenesis in vitro may have some implicit limitations, possibly due to the lack of metabolic competence of fibroblasts in activating certain chemicals (13). For example, a higher rate of benzo(a)pyrene (BP) metabolism was observed in human embryo fibroblasts mixed with 20% epithelial cells than in cultures consisting of only fibroblast (14). Since the primary tumor type in humans is carcinoma and not sarcoma, it is essential to test the carcinogenic potential of chemicals on epithelial cells.

The organ culture system of mouse prostate gland, first described by Lasnitzki (15, 16), appears to be a reliable test system for initial evaluation of the carcinogenic effect of chemicals on epithelial cells. Briefly, groups of control and 3-methylcholanthrene (MCA)-treated explants of mouse prostate were maintained in organ culture for 20 to 25 days. Alveolar epithelium of explants maintained in normal culture medium remained histologically differentiated with a relatively low rate of cellular proliferation, but the epithelium of cultures treated with MCA exhibited hyperplasia, squamous metaplasia, anaplasia and, in some cases, invasion through the basement membrane (15). The neoplastic potential of carcinogen-treated mouse prostate cultures has been demonstrated (17). Inoculation of cell suspensions prepared from the carcinogen-treated cultures into syngenic mice produced transplantable tumors whereas no tumors were formed when cell suspensions were made from untreated control explants (17). Thus the data showed that malignant transformation can be achieved in mouse prostate organ cultures and that histological changes can be used to indicate the effects of carcinogens in vitro.

In an effort to establish an in vitro system for determining the carcinogenic potential of chemicals, we have examined the ability of selected compounds (carcinogenic and noncarcinogenic) to induce hyperplasia and anaplasia in mouse prostate organ cultures. These compounds include MCA (requiring metabolic activation); the 11-12 epoxide of methylcholanthrene (Ep.MCA; an

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activated metabolite of MCA); BP; pyrene (PY); phenanthrene (Phe); and N-methyl-N-nitro-N-nitrosoguanidine (MNNG; not requiring metabolic activation). The degree of hyperplasia induced by each compound was determined by estimating changes in the cellular proliferation rates of prostate explants (18, 19).

MATERIALS AND METHODS

The organ culture technique used was a modification of Lasnitzki's method (15–16). Eight- to 12-week-old BDF_1 or C3H mice were killed instantaneously by exposure to carbon dioxide (dry ice), and the ventral prostates were aseptically removed. The prostate tissue was freed from fatty tissue and teased into small explants (1.5-mm by 1.5-mm). Precautions were taken to avoid a regenerative response which could be caused by excessive damage to the tissue during the process of making explants. Six to eight explants were placed on the bottom of a scratched, 35-mm plastic Petri dish. Two ml of CMRL 1066 medium supplemented with 10% fetal bovine serum (FBS) or horse serum (HS) (Grand Island Biological Co.) 100 IU per ml each of penicillin and streptomycin, and 25 μg per ml amphotericin B were added to each dish. The cultures were gassed with a mixture of 95% O_2 and 5% CO_2 at 37°C in a sealed chamber, which was gently rocked 10 to 12 times per min to allow, alternatively, the contact of tissue with both gas and medium. Culture medium was renewed every 48 hr.

The test compounds—BP, PY, Phe, MCA, MNNG (Sigma Chemical Co.) and Ep.MAC (supplied by Dr. Selkirk of Oak Ridge National Laboratory)—were dissolved first in reagent grade acetone or dimethylsulfoxide (DMSO) at a concentration of 4 mg per ml. Portions of the aceton solution were mixed with FBS or HS to give different concentrations of a compound. Aliquots of serum suspensions (50 μl) then were added to each culture dish (2 ml medium). Fresh solutions of the test compounds were prepared at each renewal of the culture medium every 48 hr. The final concentration of acetone or DMSO in the culture medium was approximately 0.05%. Equivalent amounts of acetone, DMSO, FBS or HS also were added to the control cultures. All experiments were carried out in subdued light.

The degree of hyperplasia induced by carcinogens at various intervals after the treatment was determined by estimating mitotic indices, using the colcemid metaphase arrest technique (18, 19). Since colcemid arrests only those cells entering metaphase without influencing the progression of cells through the cell cycle, treatment of cultures with colcemid over a fixed duration gives an estimation of the number of dividing cells during that period. Four hr prior to termination of cultures, colcemid (2 μg per ml) was added to each culture dish. In one experiment, eight control and MCA-treated explants also were pulse-labeled for 2 hr with tritiated thymidine (H^3TdR, 2 μCi per ml, sp. act. 6.7 Ci per mMol) at 8 days after exposure to the carcinogen. Cultures were fixed in buffered formalin and processed for histology. Five-μm sections were cut and stained in hematoxylin and eosin. For autoradiography, 4-μm sections were obtained, dipped in Kodak NTB2 emulsion, exposed for 2 weeks in the dark at 4°C, developed in Kodak D-19 developer and stained in hematoxylin and eosin.

The mitotic index (MI) in each explant was determined by counting the number of arrested metaphases in 500 to 1000 cells per explant. Two sections from each explant, eight to 10 sections per group, were scanned and MI ± SD calculated for each group. The significance of the difference between the control and experimental groups was determined by the Student's "t" test. A P value of <0.05 was considered significant.

EXPERIMENTS AND RESULTS

Effect of different concentrations of carcinogenic and noncarcinogenic compounds on MI in the prostatic epithelium in vitro. The effect of each compound was examined in separate experiments. For each experiment, groups of cultures were prepared and treated with various compounds at different concentrations. Eight days after the treatment, colcemid (2 μg per ml) was added to both the control and experimental cultures, and the explants were fixed 4 hr later. Mitotic indices were determined in the prostatic epithelium.

Table 1 shows that all known carcinogens tested in this study (MCA, Ep.MCA, BP, MNNG) stimulated proliferation while the noncarcinogens (PY, Phe) had no effect. The mitotic stimulatory effect of the carcinogens was concentration-dependent, the maximum stimulation being produced at 1, 2, 4 and 8 μg per ml of MNNG, Ep.MCA, MCA and BP, respectively. Therefore in all other experiments the carcinogens were added to the cultures at these concentrations.

Effects of carcinogens on MI at various intervals after treatment. Prostate cultures were prepared and treated separately with MCA,

TABLE 1

EFFECT OF DIFFERENT CONCENTRATIONS OF CARCINOGENIC AND NONCARCINOGENIC COMPOUNDS ON MITOTIC INDEX (MI±SD)[a] IN MOUSE PROSTATE EXPLANTS AT 8 DAYS AFTER TREATMENT IN VITRO

Compound[c]	Concentration μg/ml[b]					
	0	0.5	1.0	2.0	4.0	8.0
MCA	211±33	287±85	496±97[d]	531±199[d]	1343±170[e]	
Ep.MCA	95±2	462±218[e]		508±223[e]		
BP	154±54		342±181		537±179[e]	834±289[e]
PY	70±6		79±28		62±10	87±19
Phe	62±30		62±17		56±14	58±15
MNNG	162±27	327±84	693±178[e]			

[a] MI±SD is the number of arrested metaphases per 10^5 cells during a 4-hr period of colcemid treatment.
[b] Molecular weights: MCA = 268; Ep.MCA = 284; BP = 252; MNNG = 133.
[c] 3-Methylcholanthrene (MCA); 11-12 epoxide of methylcholanthrene (Ep.MCA); benzo(a)pyrene (BP); pyrene (PY); phenanthrene (Phe); N-methyl-N-nitro-N-nitrosoguanidine (MNNG).
[d] Mitotic stimulation statistically significant ($P < 0.05$) as compared to the corresponding control values.
[e] Mitotic stimulation statistically significant ($P < 0.01$) as compared to the corresponding control values.

Ep.MCA, BP or MNNG. At 4, 8 and 12 days after the treatment, colcemid was added to the control and carcinogen-treated cultures, and they were fixed 4 hr later. The MI was determined in the epithelium and results are presented in Table 2. It shows that the carcinogens stimulated proliferation in the epithelium at 4 days after the treatment. Eight days after the treatment, MCA, Ep.MCA, BP and MNNG stimulated cellular proliferation by 332%, 141%, 441% and 302%, respectively, as compared with the control values. Similar hyperproliferative activity was present in the carcinogen-treated explants at 12 days after the treatment. It should be noted that among the compounds examined, MNNG appears to be the most potent carcinogen to produce hyperplasia in the mouse prostate epithelium, since it produced a high mitotic stimulatory effect at a very low concentration.

Morphological changes in the prostatic epithelium induced by carcinogens. Prostate cultures were continuously treated with MNNG, Ep.MCA, MCA or BP at concentrations of 1, 2, 4 or 8 μg per ml, respectively, and were examined for hyperplasia and anaplasia at 4, 8 and 12 days after the treatment. The corresponding, untreated cultures were examined also.

The alveolar epithelium of the control explants loses its grandular appearance and the epithelium folds characteristic of prostate gland in vivo disappear. The epithelium of the control explants through a 4- to 12-day culture period remains low and shows negligible secretory activity. The alveoli are lined with one to two layers of epithelial cells (Fig. 1) with little proliferative activity, as indicated by the sparse H^3TdR labeling (Fig. 2). Some newly formed alveoli are present at the periphery of the control explants.

Treatment of the explants with carcinogens (MCA, for example) stimulated proliferation in the prostatic epithelium as early as 4 days after treatment. The hyperplasia became extensive at 8

TABLE 2

PROLIFERATIVE RESPONSE (MI±SD)[a] OF PROSTATIC EXPLANTS TO CARCINOGENS AT DIFFERENT INTERVALS AFTER TREATMENT IN VITRO

Carinogen[b]	Days After Treatment					
	4		8		12	
	Control	Treated	Control	Treated	Control	Treated
MCA (4 μg/ml)	290±78	671±256[c]	91±42	394±147[c]	106±39	194±64
Ep.MCA (2 μg/ml)	198±54	485±84[c]	158±81	381±75[c]	58±30	243±24[c]
BP (8 μg/ml)	—	—	154±54	834±289[c]	197±55	655±189[c]
MNNG (1 μg/ml)	64±23	196±34[c]	50±14	201±35[c]	54±26	209±47[c]

[a] MI±SD is the number of arrested metaphases per 10^5 cells during a 4-hr period of colcemid treatment.
[b] 3-Methylcholanthrene (MCA); 11-12 epoxide of methylcholanthrene (Ep.MCA); benzo(a)pyrene (BP); N-methyl-N-nitro-N-nitrosoguanidine (MNNG).
[c] Mitotic stimulation statistically significant ($P < 0.05$) as compared to the corresponding control values.

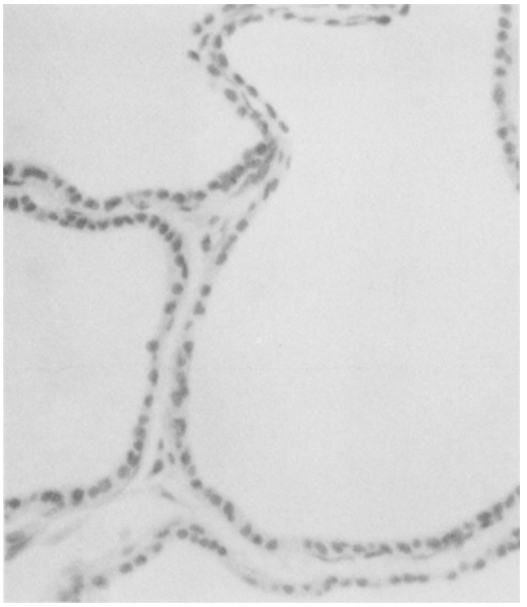

FIG. 1. Light micrograph of mouse prostate explant maintained in organ culture for 8 days. ×200.

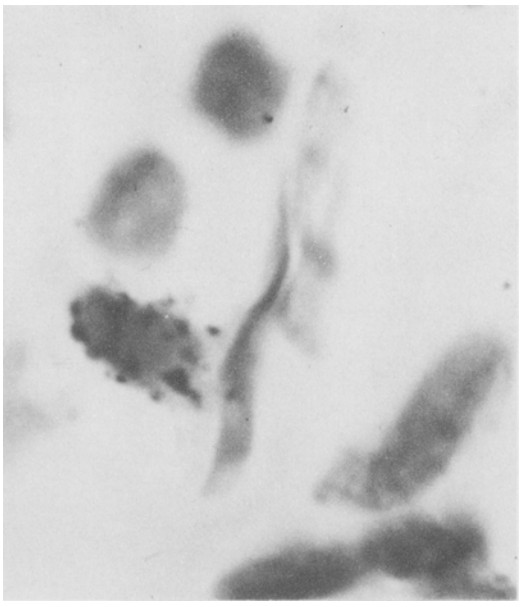

FIG. 2. Autoradiograph of prostate explant maintained in organ culture for 8 days. ×1000.

days as evidenced by the large numbers of H^3TdR-labeled and mitotic cells in these explants (Fig. 3). In contrast to the control explants where mitotic cells were localized only in the basal epithelial layer, carcinogen-treated explants contained mitotic cells above the basal layer. The epithelium of treated explants became five to six cell layers thick and exhibited a high degree of proliferative activity (Fig. 4). The degree of hyperplasia in the treated explants was variable. For example, only 70 to 75% of the treated explants responsed to the carcinogen treatment. Among the affected explants, 50 to 60% showed hyperplastic changes in 80 to 95% of the alveoli whereas in the remaining 40 to 50% of the explants, only 30% of the alveoli were affected. Frequently, only part of the alveolar wall was involved whereas the remainder of the epithelium still consisted of one to two layers of cells. By 12 days the hyperplastic alveoli were pleomorphic and cells were polygonal with large irregular basophilic nuclei. At this time, foci consisting of anaplastic cells of irregular shape and size were present. Similar hyperplastic and anaplastic changes in the prostatic epithelium was observed in explants treated with Ep.MCA, BP or MNNG.

Effect of withdrawal of carcinogens on MI. Carcinogen was withdrawn after 8 days of initial treatment. Three groups of explants (3 to 4 dishes per group) were set up for each experiment with a different carcinogen:

GROUP 1. Untreated control cultures—fixed on days 8, 9, 10 and 12 after their initiation.

GROUP 2. Cultures treated with carcinogens (MCA, MNNG or Ep.MCA)—fixed on days 8, 9, 10 and 12 after the treatment.

GROUP 3. Cultures were treated with carcinogen. After 8 days, carcinogen was withdrawn, explants rinsed twice in control medium and reincubated in control medium. They were fixed 24, 48 and 96 hr later.

The MI was determined in various groups of explants and the results are shown in Table 3. As expected, explants which were treated continuously with MCA, Ep.MCA or MNNG for the entire duration of the experiment showed hyperplasia of the alveolar epithelium (Group 2). Withdrawal of carcinogen from the cultures after 8 days of treatment had no effect on hyperproliferation, at least for 96 hr (Group 3). In fact, hyperplasia of the explants in Group 3 appeared more pronounced and some alveoli showed squamous metaplastic changes at 96 hr after withdrawal of the carcinogens. In these cultures the alveolar epithelium remained five to six cell layers thick and many mitotic cells were still present (Fig. 5). No significant differences in the values of MI was observed between the explants of Groups 2 and 3 (Table 3).

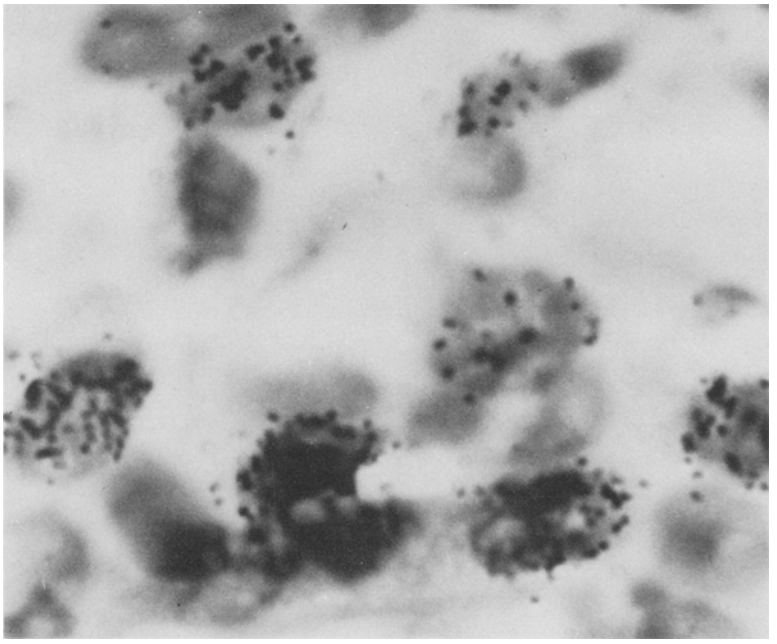

FIG. 3. Autoradiograph of prostate explants treated with MCA (4 μg per ml) for 8 days in vitro. ×1000.

DISCUSSION

The organ culture system described in this study shows that the mouse prostate tissue can be maintained for sufficient time to determine if a chemical will have a potential to induce preneoplastic hyperplasia and anaplasia in vitro. The degree of activity of the carcinogen was determined by the degree of hyperplasia produced. The results show that the untreated control cultures

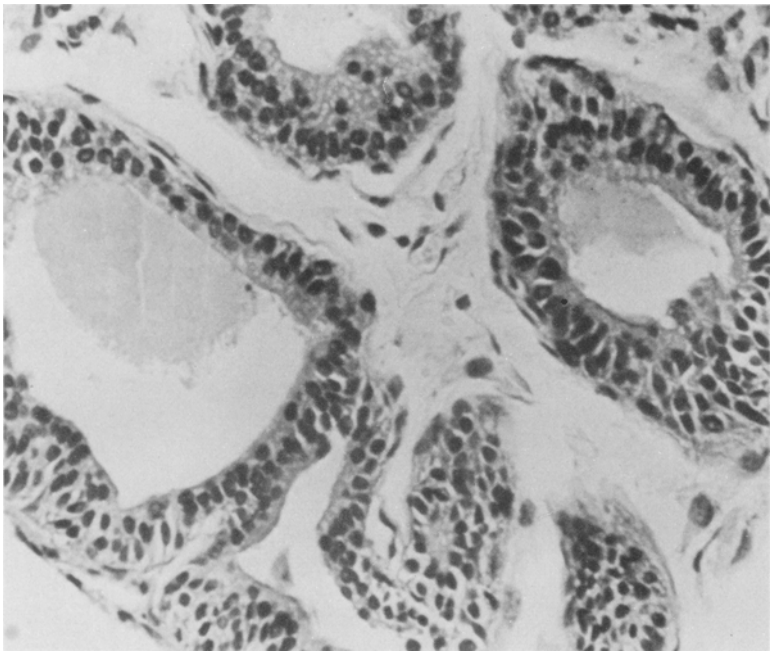

FIG. 4. Light micrograph of prostate explant treated with MCA (4 μg per ml) for 8 days in vitro. ×300.

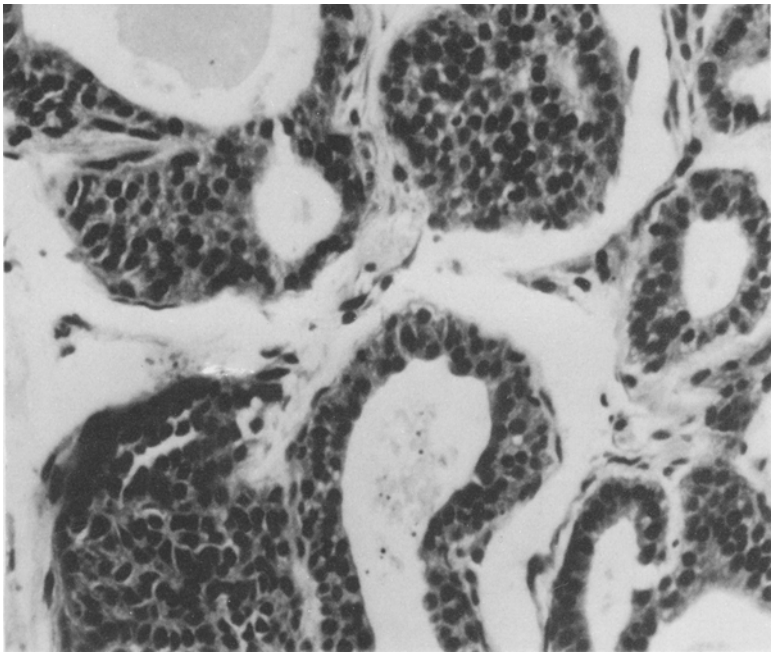

FIG. 5. Light micrograph of prostate explant treated with MCA (4 μg per ml) for 8 days followed by incubation in control medium for an additional 96 hr. ×300.

maintained their in vivo appearance, in that most alveoli remained lined with one to two layers of columnar epithelial cells which exhibited little mitotic activity. This normal architecture of the prostate gland was maintained for at least 12 days, after which time flattening of the epithelium was observed.

All four carcinogens examined produced hyperplasia and some degree of squamous metaplasia after 12 days of treatment. However, noncarcino-

TABLE 3

EFFECT OF WITHDRAWAL OF MCA, MNNG OR EP.MCA ON MITOTIC INCIDENCE (MI±SD)[a] IN MOUSE PROSTATIC EPITHELIUM IN VITRO

Days After Treatment[b] / Hr. After Withdrawal of Carcinogen[c]		8	9 24	10 48	12 96
MCA 4 μg/ml	Group 1[d]	123±56	187±87	34±8	34±15
	Group 2	290±29[e]	448±54[f]	308±98[f]	318±76[f]
	Group 3		536±150[f]	229±48[f]	225±50[f]
MNNG 1 μg/ml	Group 1	58±13	169±23	279±141	137±62
	Group 2	297±73[e]	338±69[e]	737±75[f]	399±94[e]
	Group 3		465±38[e]	748±70[f]	500±115[f]
Ep.MCA 2 μg/ml	Group 1	137±40	160±65	162±65	158±53
	Group 2	220±40[e]	883±191[f]	416±48[e]	350±70[e]
	Group 3		485±81[e]	439±35[f]	427±65[f]

[a] MI±SD: Number of arrested metaphases per 10^5 cells during a 4-hour period of colcemid treatment.

[b] Carcinogen was withdrawn after 8 days of treatment and cultures reincubated in the control medium for an additional 24, 48 and 96 hr.

[c] 3-Methylcholanthrene (MCA), N-methyl-N-nitro-N-nitrosoguanidine (MNNG); 11-12 epoxide of methylcholanthrene (Ep.MCA).

[d] Group 1. Untreated controls.
Group 2. Cultures continuously treated with the respective carcinogen.
Group 3. Cultures were treated with the carcinogen. After 8 days, the respective carcinogen was withdrawn and cultures reincubated in the control medium and fixed at 24, 48 and 96 hr later.

[e] Mitotic stimulation statistically significant ($P < 0.05$) as compared to the corresponding control values.

[f] Mitotic stimulation statistically significant ($P < 0.01$) as compared to the corresponding control values.

genic hydrocarbons, PY or Phe, had no effect on prostate cultures. The degree of hyperplasia produced by each carcinogen was concentration-dependent although different concentrations of MCA, Ep.MCA, BP or MNNG were required to produce maximum hyperplasia (Table 1). This difference in concentration probably is caused by a number of factors including (a) the permeability of the cell membranes to the carcinogen, (b) ability of the carcinogen to bind to cellular macromolecules, (c) stability of the carcinogen, and (d) mechanism of action of the carcinogen.

It was of interest to note that in explants exhibiting extensive hyperplasia, all alveoli were not involved. The normal-looking alveoli lined with one to two layers of epithelial cells often were located adjacent to the hyperplastic alveoli. Furthermore, even within the same alveolus, only part of the epithelium was hyperplastic, and the remainder of the epithelium looked quite normal. Similar observations have been reported by Lasnitzki (15). This diversity of response within the same explant may be due either to the availability of the carcinogen or to variable sensitivity of the prostate cells to the carcinogen. However, since the normal and hyperplastic alveoli were present in the same region, it would appear that some cells remained resistant to the carcinogen action. The refractory cells may have been at a particular stage of differentiation pathway from which they could not be reverted to the growth phase. The reason for failure of 25 to 30% of explants to respond to carcinogens is not understood.

Withdrawal of MCA, Ep.MCA or MNNG after 8 days of treatment and reincubation of the treated explants for an additional 96 hr produced no reduction of hyperplasia. There were no differences in the values of MI between explants which continued to receive the carcinogen and those from which the respective carcinogen was removed. In both groups, the MI were higher as compared with the corresponding control values. Similar observations of the persistence of hyperplasia after the withdrawal of carcinogen also has been reported in hamster tracheal organ culture (20). The presence of hyperplasia in explants from which carcinogens have been withdrawn probably is not due to the accumulation of sufficient carcinogens or their relatively stable metabolite. For instance, MNNG has a half-life of approximately 30 min at pH 7.4 (21). In the case of MCA, 80% of the carcinogen is lost from tissue within 48 hr after its withdrawal (22). Moreover, intermediates derived from the activated carcinogens are very unstable (23). The most likely explanation for the persistent hyperplasia is that the carcinogens induce relatively permanent alterations in the target macromolecules (DNA or proteins) of cells so that synthesis of enzymes required for hyperplasia continue (24) until it is switched off by some antagonizing factors (18).

Although carcinogens have been shown to induce hyperplastic and anaplastic changes in the mouse prostate explants, only limited information is available as to whether the carcinogen-treated explants will produce tumors on implantation into animals. Studies to assess the neoplastic nature of these explants by implanting into syngenic or immunorepressed animals are under consideration. Heidelberger and Iype (17) have demonstrated that inoculation of cell suspensions prepared from MCA or dimethylbenz(a)anthracene-treated prostate explants into syngenic animals produced transplantable sarcomas and carcinomas.

In summary, the mouse prostate organ culture system may provide a method to test for the carcinogenic potential of chemicals. The known carcinogenic chemicals tested in this study induced "preneoplastic" hyperplasia whereas the inactive compounds did not. Furthermore, the relative activity of a carcinogen may be evaluated according to the degree of hyperplasia produced.

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