CHEMICAL CARCINOGENS AND RESPIRATORY EPITHELIUM

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1. Introduction

In an earlier communication [1], carcinogenic hydrocarbons benzo(a)pyrene (BaP), 7,12-dimethylbenz(a)anthracene (DMBA) and 3-methylcholanthrene (MC) were shown to produce metaplasia and increased proliferation of the epithelium of suckling rat tracheas maintained in organ culture. Tissues exposed for several days by including hydrocarbon in the medium, and tissues so treated and then withdrawn to hydrocarbonfree media were examined at intervals by histologic and autoradiographic methods; the latter were applied to tissues fed tritiated thymidine to detect cells synthesizing DNA. All hydrocarbons caused extensive death of connective tissue elements (fibrocytes, cartilage) and led to thymidine incorporation in only a small proportion of surviving connective tissue cells.

Distinctive epithelial alterations were produced by each hydrocarbon, but the general effect of all three agents was reduction in numbers of differentiated cells, marked increase in proportions of basal cells incorporating tritiated thymidine and slightly increased proportions of cells in mitosis (table I). Cells

TABLE I

COMPARISON OF RATES OF MITOSIS AND OF ^{3}H-THYMIDINE INCORPORATION IN RAT TRACHEAL EPITHELIUM EXPOSED TO DIMETHYLBENZANTHRACENE IN ORGAN CULTURE
(Modified from table 3 in [1].)

Expt. No.	DMBA Days Exposed and Dose	Total Cells per Circumference: Controls Labeled %	Controls Mitotic %	Treated Labeled %	Treated Mitotic %	Description of Epithelial Height
34	6 d, 2.0μg/ml	5.0	0.4	23.4	1.6	Very high
9	7 d, 1.4μg/ml	5.6	0.9	4.7	1.3	Medium high
8	9 d, 0.8μg/ml	2.9	0.5	23.6	0.4	Low
8, 13	9 d, 1.4μg/ml	7.5	—	15.4	0.9	Low
13	11 d, 1.4μg/ml	7.1	0.7	38.7	1.5	Low
	Means	5.5	0.6	21.1	1.1	
	Ratio L/M	10/1		20/1		

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of irregular shape and staining characteristics appeared above the basal cells. Since these cells took the place of normally differentiated columnar cells and were highly variable in appearance, they were considered to be "metaplastic," that is, changed to a form not normal to the tissue.

Epithelial metaplasia was replaced by highly differentiated columnar epithelium upon withdrawal from DMBA but not from BaP. Both hydrocarbons produced persistent increase in the proportion of basal cells and in the proportion of basal cells incorporating tritiated thymidine after withdrawal of the hydrocarbons [1].

None of the hydrocarbons increased the proportion of dividing cells to the same degree as they increased the proportion of cells synthesizing DNA during the period of these observations, indicating that preparation for cell division was increased while actual cell division was blocked, at least for a time.

Significant epithelial changes occurred, therefore, after only a few days of continuous exposure to hydrocarbons and some of these were reversible in an equally short time. The issue thus arose as to the nature and sequence of cellular actions of carcinogenic hydrocarbons and as to what relation these have to carcinogenic transformation.

The present report adds new data on penetration of labeled BaP into columnar and basal cells. In addition, more closely spaced observations following a single brief exposure to DMBA are reviewed [2]. These observations explore the sequence of events occurring in respiratory epithelium upon first contact with carcinogenic hydrocarbons. The object of this communication is to interpret these observations and set up hypotheses for future experimental evaluation.

2. Materials and methods

2.1. *Source and preparation of tissue.* Suckling rats one to five days of age were obtained from a breeding colony of the Nelson strain. Animals were anesthetized by chilling at 5° C for 10 to 15 minutes, pinned out while motionless but breathing, and the skin cleaned with 70 per cent alcohol. After death by bleeding from the abdominal aorta, the trachea could be removed without contamination with blood and transferred to Tyrode's solution. Tracheas were used as single explants, so placed on a rayon mesh as to permit future orientation of the axis of the lumen and to retain the identity of the animal of origin.

2.2. *Culture system.* Rooster plasma, collected without anticoagulant, was stored at 4° C for not more than two weeks. Chick embryos 12 days of age were minced with scissors, and the mince was diluted with an equal volume of Tyrode's solution and centrifuged lightly to yield an opalescent, slightly turbid embryo extract which was stored not more than a day. A clotted medium was prepared in plastic organ culture dishes and was composed of 50 per cent plasma, 25 per cent embryo extract and 25 per cent commercially prepared frozen chick serum. The small plastic dishes bore a water soaked ring of filter paper to provide a moist chamber. Dishes were enclosed in a box fitted for gassing and received a

humidified mixture of 3 per cent CO_2 to 97 per cent O_2 at about 100 ml per minute for 30 minutes daily.

Each petri dish contained about 1.0 ml of medium which had a surface diameter of about 2.5 cm; this surface received two strips of rayon mesh, each carrying three to five explants. Explants became adherent to the mesh; they were rinsed free of lysed clot and mucus, and the mesh with attached explants was transferred to fresh media about every second or third day. This is essentially the system developed by Fell and Robison [3] as modified by Shaffer [5]. Gassing followed the method described by Trowell [6].

2.3. *Addition of hydrocarbons and radioisotopes.* Acetone solutions of 7,12-dimethylbenz(a)anthracene (DMBA), benzo(a)pyrene (BaP), benzo(e)-pyrene (BeP) or of 3-methylcholanthrene (MC) were prepared without further purification of the products. Hydrocarbon solutions were added to chicken serum to produce "stock suspensions" of such composition that one part of stock suspended in 30 parts of medium gave final concentrations of hydrocarbon from 0.2μg to 16μg/ml and concentrations of acetone not greater than 0.2 per cent.

Tritiated thymidine was diluted in Tyrode's solution to 125μc/ml. At the end of a period of incubation, each explant received a drop (about 0.02 ml) of the isotope solution for two minutes. This was removed with a Pasteur pipette, and after reincubation for one half hour, the explants were washed repeatedly in Tyrode's solution before fixation.

2.4. *Histologic preparations.* Explants attached to the rayon mesh were fixed for one half hour in Bouin's solution and dehydrated. The rayon mesh was trimmed to separate each explant with its surrounding mesh, and the mesh was dissolved during final dehydration in acetone prior to clearing in toluene and embedding in paraffin. Serial sections were examined histologically after staining with hematoxylin and eosin or Mowry's colloidal iron. Sections from tissue labeled with tritiated thymidine were prestained by the Feulgen method after eight minutes hydrolysis before autoradiography by the stripping film technique of Pelc [4] or by dipping in undiluted Kodak NTB2.

Tritiated BaP (^{3}H-BaP) was added to organ cultures by either of two schedules. Freshly planted cultures were maintained for three days on normal media, then transferred to medium containing labeled hydrocarbon and fixed after intervals up to 5 days. Cultures were maintained on control media, or media containing BeP or unlabeled BaP for 11 days, then moved to medium containing ^{3}H-BaP and fixed after intervals up to six hours. The two procedures were aimed at distinguishing between incorporation of label from the hydrocarbon by previously unexposed tissue and by tissue in which handling of the label might in some degree be altered by prior exposure to hydrocarbons.

Tissues exposed to ^{3}H-BaP were fixed and processed for autoradiography as described above; this meant that fat solvents would have removed all label except that closely bound in tissue either by biologic complexing or by such bonds as might be produced during tissue denaturation by Bouin's fixative.

Autoradiographs of tissue exposed to ^{3}H-BaP were made on histologic sections prestained by the Periodic Acid-Schiff reagent and stained, after development of autoradiographs, with hematoxylin. Such preparations permitted identification of cell type and distinctions between nuclear and cytoplasmic labeling. Quantitative counts of photographic grains over cell areas were performed at 1000× magnification with an ocular grid. Grains were scored over cytoplasmic or nuclear zones in fields delimited by the grid.

Background grains were counted in ten times as many grid areas as were counted in adjacent tissue areas, an average background per grid area was established and subtracted from the counts over the nearby tissue zones to render counting values technically uniform.

3. Results

3.1. *DNA synthesis in DMBA treated explants.* Autoradiography was undertaken on sections from tracheas exposed to tritiated thymidine for 30 minutes before fixation at three hours or at 1, 2, 3, 4, 6 and 7 days after brief treatment with DMBA at 16μg/ml. The proportion of labeled epithelial cell nuclei reached a maximum at one to two days and had returned to control levels by four to six days after exposure (table II). No loss of columnar cells preceded the increased

TABLE II

DNA Synthetic Activity and Morphologic State in Tracheal Epithelium of Suckling Rats Exposed to DMBA in Organ Culture

Three day cultures exposed to 10 to 16 g DMBA/ml for 30 minutes (from [2]).

Time on Normal Medium after DMBA	Autoradiographic Observations			Morphologic Observations
	Treated Explant			
	Total Epithelial Cells per Section (mean)	Per Cent Labeled with ^{3}H-Thymidine (mean)	Ratio Per Cent Labeled, Treated/Control	Predominant Epithelial State in Treated Explants
3 hrs.	812	3.7	1.4	normal
1 day	609	20.0	6.1	basal and columnar hyperplasia
2 days	643	16.9	9.4	as above
3 days	410	10.2	2.4	as above
4 days	420	6.7	1.4	high metaplasia
6 days	674	3.1	1.3	columnar hyperplasia
7 days	688	4.8	0.43	metaplasia; basal and columnar hyperplasia

labeling of basal cells and instead there was unusual elongation of columnar cells which were pseudostratified. This detail in the response to DMBA demonstrated that the earliest cellular effect of penetration to the epithelium occurred

in one day, that stimulation of replication occurred before columnar cell death and that this feature of the action of DMBA was temporary if an appropriate low concentration was applied once.

The peak of the effect of DMBA on replicative activity had passed by the time some metaplastic changes appeared (table II). Abnormally or incompletely differentiated cells either arose from basal cells to replace the unusually high columnar cells, or columnar cells underwent modulation from differentiated to metaplastic states.

3.2. *Distribution and rate of appearance of label in organ cultures of rat trachea exposed to tritiated benzo(a)pyrene.*

3.2.1. *Labeling in fresh explants.* Organ cultures were transferred at three days to media containing ^{3}H-BaP, 0.520 C/mM, 6μg/ml. Autoradiographs revealed variable distribution of label over the different cell types of the tracheal wall and revealed differences in the time of appearance of label in each type of

TABLE III

DISTRIBUTION OF LABEL IN AUTORADIOGRAPHS OF SECTIONS OF SUCKLING RAT TRACHEA AFTER CONTINUOUS EXPOSURE TO TRITIATED BENZO(A)PYRENE IN ORGAN CULTURE

The unit area was that zone of a microscope field enclosed within an ocular grid of constant size under uniform magnification (about 1000×). Values shown are average of counts in 70 to 120 unit areas for the explants representing each day. Background has been subtracted.

Histologic Sites in Sections of Tracheal Wall	Grains Counted per Unit Area		
	Day 1	Day 3	Day 5
1. Mucus on luminal surface of epithelium	9.9	15.2	19.8
2. Differentiated epithelium:			
a. Columnar cells—			
nucleus and cytoplasm	10.4	5.0	7.8
nucleus only	3.2	2.4	3.4
b. Basal cells—			
nucleus and cytoplasm	0.7	0.0	7.0
3. Undifferentiated epithelium:			
a. Migratory cells—			
nucleus and cytoplasm	none present	41.3	60.8
nucleus only	none present	22.8	17.5
4. Connective tissues:			
a. Subepithelial	11.4	28.1	41.2
b. Between cartilages	15.2	28.2	26.4
c. External	none present	45.2	64.0
5. Cartilage:			
a. Matrix	0.0	0.3	1.8
b. Cell body	0.0	4.4	5.5
6. Macrophages (presumptive)	none present	13/cell	40/cell

cell (table III). Quantitative estimates of grain number over specific cells and even over portions of cells were made at high magnification by use of an ocular grid to delimit unit areas. Means from counts in large numbers of such grid areas (table III) demonstrated the following.

(1) Label appeared at maximal concentration over columnar epithelium (table III, item 2) on day one. Cytoplasm was more heavily labeled than nucleus. These values fail to demonstrate that label was concentrated over goblet cells by day three, and the increasing label in shed mucus (table III, item 1) supports the conclusion that label became associated with intracellular mucous goblets and was discharged by goblet cells still capable of releasing such labeled mucus.

(2) *Basal cells did not label significantly until the fifth day.* This is important because in analogous experiments with unlabeled hydrocarbon in which ^{3}HT was used to detect the earliest activation of DNA synthesis, increased proportions of basal cells incorporated ^{3}HT after one day of exposure to hydrocarbon (table II). These findings strongly suggest that basal cell replication begins indirectly as a result of action of the hydrocarbon on columnar cells rather than directly as a result of penetration to basal cells.

(3) Connective tissues labeled with increasing intensity during five days' exposure to tritiated BaP (table III, item 4). Cartilage (table III, item 5) acquired label slowly and to a low degree, with cell bodies more heavily labeled than matrix.

(4) Massive accumulations of label appeared at three and five days over cells scattered randomly in connective tissues. These are presumably macrophages which are known to sequester hydrocarbons in quantity (table III, item 6).

(5) The more rapidly growing cells incorporated larger amounts of label than the more stable cell groups. Connective tissue on the exterior of the explant (table III, item 4, External), and the epithelium migrating on to the exterior of explants (table III, item 3) were more heavily labeled than the same tissues within the organized structures nearer the lumen. Although a diffusion effect was anticipated since more label would be available to cells nearer the medium, migratory cells were heavily labeled even at sites most remote from the substrate medium.

3.2.2. *Labeling in explants pretreated with hydrocarbons.* Organ cultures were maintained for 11 days on media containing BeP, BaP, or acetone vehicle only. Cultures were transferred to media containing tritiated BaP at the concentration described in the preceding section. Cultures were fixed at 1, 3 or 6 hours, sectioned and examined autoradiographically for distribution of label (table IV). Semiquantitative grading of label demonstrates that hydrocarbon treated tissues acquired slightly more label and did so slightly earlier than control tissue. The difference between BaP and control tissues was greater than BeP and control, and it is likely that cultures treated with BeP were not different from control cultures.

Migratory epithelial cells in all cultures incorporated more label as in section 3.2.1, above. The luminal epithelium which had become metaplastic after 11 days' exposure to BaP labeled more heavily with tritiated BaP than did control epithelium. Thus, more labeled hydrocarbon entered epithelial cells which were undifferentiated and which have in the past been found to be in DNA synthesis after BaP treatment.

TABLE IV

LABELING WITH TRITIATED BENZO(A)PYRENE IN SUCKLING RAT TRACHEAS PREVIOUSLY MAINTAINED ON ORGAN CULTURE MEDIA CONTAINING UNLABELED BeP OR BaP

COMMENTS.

(1) Migratory epithelial cells on the exterior of the explant are rapidly growing and undifferentiated; these label faster and to a higher degree in all systems.

(2) Columnar (or metaplastic) cells and basal cells in the epithelium proper were most highly labeled in tracheas previously exposed to BaP; this hydrocarbon increases the number of cells in DNA synthesis and reduces differentiation.

(3) Connective tissue labeling was not affected by the prior mode of cultivation, but cartilage labeled more quickly and to a greater extent after BaP than after BeP, though both exceeded control levels.

Histologic Sites in Sections of Tracheal Wall	Duration of Labeling with Tritiated BaP after 11 Days' Cultivation with Hydrocarbons								
	Control			BeP			BaP		
	1 hr	3 hr	6 hr	1 hr	3 hr	6 hr	1 hr	3 hr	6 hr
	(Semiquantitative counts of grains per area)								
Differentiated Epithelium									
Columnar cells	0	±	1	0	3	1	0	1	4
Basal cells	0	0	0	0	0	±	0	±	2
Undifferentiated Epithelium									
Migratory cells	1	5	15	0	3	15	1+	6	17
Basement Membrane	0	0	1	0	1	1	0	0	3
Connective Tissues	0	4	3	0	5	4	1	2	3
Cartilage Cells	0	1	1	0	1	4	0	4	7
Cartilage Matrix	0	2	2	0	2	3	±	3	1
Totals	1	12	23	0	15	28	2	17	37

Connective tissues labeled equally in all systems. Cartilage incorporated more tritiated hydrocarbon in cultures pretreated with hydrocarbon.

4. Discussion

4.1. *Premature proliferation of basal cells.* Data summarized in table II indicate that a proliferative response, that is, increased proportions of cells labeling with ^{3}HT presumably on behalf of DNA synthesis, occurs among basal cells before the differentiated columnar cells are killed by exposure to DMBA. Data summarized in tables III and IV demonstrate prompt penetration of label from a different hydrocarbon (^{3}H-BaP) with principal early localization in cytoplasm of columnar cells. These two findings were made with different hydrocarbons. For this reason the interpretations made now must be provisional, but previous data [1] demonstrated comparability between the two hydrocarbons in that both are toxic for connective tissues and induce metaplasia and proliferation in epithelial cells at about comparable times after onset of exposure.

The central feature in the findings described above is that hydrocarbon reached nondividing differentiated columnar cells, did not kill them but instead preserved them in place for several days and even caused the columnar epithelium to become unusually high or pseudostratified. While this state of columnar cells was being induced basal cells began to proliferate, a process usually resulting from death of differentiated columnar cells. The sequence of these events indicates that proliferation of basal cells was premature.

Two explanations are possible.

First, basal cells were directly stimulated to proliferate. Most early effects of hydrocarbons are toxic [1], [2] rather than stimulating to cellular processes and basal cells acquired little label from ^{3}H-BaP at an early time, suggesting that this cell type was initially protected, for some reason, from any direct action of the hydrocarbon. Evidence for an eventual toxic effect on proliferating basal cells is presented in section 4.2, below. Primary stimulation of basal cell proliferation is, therefore, not the most likely cause of premature proliferation.

Second, basal cells were stimulated indirectly. That is, basal cells began to proliferate due to action of the hydrocarbon at a site other than within basal cells. Basal cells are normally restrained from proliferating by the presence of differentiated cells. This restraint was apparently lifted even when differentiated cells were present. The restraining power of differentiated cells may, therefore, have been lost coincident with the visibly high content of label from ^{3}H-BaP in columnar differentiated cells. The resultant increase in basal cell proliferation could thus be regarded as a physiologic response of basal cells, namely, proliferation to replace columnar cells because of lack of restraining "signals" from columnar cells.

These interpretations apply to the findings made within one and two days after beginning the exposure to hydrocarbons. Upon continuous exposure columnar cells were replaced by metaplastic cells by day six while basal cell proliferation continued; see section 4.2, below. At this point, the absence of differentiated cells would be adequate reason for continued basal cell proliferation which could still be considered to be physiologic, that is, aimed at replacement of absent differentiated cells.

4.2. *Toxic effects of hydrocarbons on proliferating basal cells.* Table I described thymidine incorporation and mitotic rates in epithelium of tracheas exposed to DMBA continuously for six to eleven days. Both labeling and mitotic rates were increased in basal cells and this is interpreted in the foregoing section as a physiologic response to loss of columnar cells. The labeling rates of treated epithelia were increased proportionately more than the mitotic rates, however, since the ratio of mean labeling rate to mean mitotic rate rose from the control value of 10:1 to 20:1.

The disproportion between labeling and mitotic rates was regarded, in the introduction, as evidence that cells preparing for division were blocked from dividing. This suggests a toxic effect on cells which have begun DNA synthesis. Failure of 50 per cent of this group of cells to reach the stage of division is indi-

cated by the shift in the ratio from 10:1 to 20:1. This means that the basal cells in DNA synthesis were not merely prevented from dividing but were lost and presumably killed if the usual interpretations regarding the mitotic cycle apply in this system. Current studies are, therefore, being made as to the association between DNA synthesis and hydrocarbon toxicity. If death of cells at some point between onset of DNA synthesis and cell division cannot be confirmed, an alternative interpretation of the disproportionately high labeling of basal cells with tritiated thymidine may have to be found.

4.3. *Basis for incorporation of label from ^{3}H-BaP.* Label from ^{3}H-BaP was concentrated in cells of different types and conditions of cellular activity at different times during progress of cultivation or during exposure to BaP.

(a) Highest concentrations of label on days one and two during continuous exposure of fresh explants was in columnar cells. At this time, these cells were metabolically active as in maintenance of ciliary activity and in secretion of mucus. Hydrocarbon label appeared in the shed mucus (table III).

(b) Highest concentrations of label on days three and five during continuous exposure to ^{3}H-BaP were present in migratory epithelial cells and connective tissue cells which had, presumably, survived the toxic action of this hydrocarbon on this type of tissue. Labeling in macrophages may or may not be part of the process of increasing connective tissue labeling.

(c) After eleven days' exposure to unlabeled hydrocarbon, label from ^{3}H-BaP appeared in migratory epithelial cells of control and treated cultures and in the basal and metaplastic epithelial cells of BaP treated cultures.

The above categories of cell type or condition of activity have only one feature in common: protein synthesis is active. This is true either in differentiated columnar cells at an early time or in proliferating epithelial or connective tissue cells at a later time. In the case of proliferating cells, these can be identified by incorporation of ^{3}HT, as in table I and in previous experiments [1], [2]. While correlation between DNA synthesis and hydrocarbon penetration are necessary, as stated in section 4.2 above, the more general situation for hydrocarbon action may be active protein synthesis for any reason and not merely for purposes of cell replication.

5. Conclusion

Evaluation of new and previous data has led to the following hypotheses.

(1) Carcinogenic hydrocarbons enter cells which are in active protein synthesis (a) as a feature of the function of differentiated cells, or (b) as a feature of cell replication.

(2) Toxic action of the hydrocarbon is exerted in both situations, (a) to stop differentiated cells from inhibiting basal cell replication, and (b) to cause a disproportion between the number of basal cells synthesizing DNA and the number reaching actual mitosis.

These hypotheses bear on the molecular level of action of carcinogens but do

not at present bear on the carcinogenic transformation since actual neoplasia has not been produced in this system.

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