The Effect of 3-Methylcholanthrene on Rat Trachea in Organ Culture

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SUMMARY

A technic has been developed for "organ" culture of free-floating rings of rat trachea in liquid medium.

Control "organ" cultures initially showed evidence of epithelium degeneration followed by a rapid regeneration encompassing the entire surface of the rings. Within two weeks these cells differentiated into a "normal" ciliated epithelium. Mural connective tissue remained viable. Fibroblasts did not overgrow. There was low grade proliferation of chondrocytes beneath the perichondrium at the tips of the cartilaginous rings. In the cultures exposed to a noncarcinogenic or weakly carcinogenic agent, 1,2-benz(a)anthracene (2.3 \(\mu g\) per 100 ml), the epithelium regenerated as a 2-cell layer which did not differentiate further and did not show evidence of continuing proliferation. Cultures exposed to a potent carcinogenic agent, 3-methylcholanthrene (2.3 \(\mu g\) per 100 ml), showed significant changes in the form and cellular characteristics of the regenerating epithelium. These involved a striking increase in epithelial proliferation with the induction of squamous metaplasia. Metaplastic epithelium showed nodular downgrowth of basal cells with marked variation in nuclear size, shape, and staining properties. No invasion through the basement membrane was observed in cultures maintained for periods up to six weeks. These alterations in cells, in culture, were quite similar to those preceding the development of invasive carcinoma in vivo studies.

INTRODUCTION

The effect of carcinogens on the bronchial epithelium of rats and the development of bronchogenic carcinoma in animals exposed to carcinogens have been described (9-11). Examination of the sequential changes occurring in the course of malignant transformations involved serial sacrifices over a period of one year. It appeared that the morphogenesis of cancer might be further studied in an in vitro "organ" culture system. This type of system would eliminate such systemic influences as hormonal control, immunologic rejection mechanisms, vagaries of blood supply, and metabolic alteration of cancerogen by distant organs. It would, therefore, permit a more specific evaluation of cancerogen-target cell interaction. Further, in contrast to cell culture, the utilization of an architecturally intact organ culture would allow for the interpretation of changes in terms of the commonly used criteria for malignant transformation in vivo.

The technic described here involves the use of thin rings of adult rat trachea, cultured in liquid media with continuous observation for periods up to six weeks.

MATERIALS AND METHODS

Female rats of a specific pathogen-free Wistar strain (CFN strain supplied by Carworth Farms, New City, New York) weighing about 100 gm were lightly anesthetized with ether and their tracheas aseptically exposed and removed. Each trachea was immediately placed in a 100 x 15 mm Petri dish containing Hank's balanced salt solution at 37°C and taken into a sterile room. The trachea was rinsed by successive transfer through 3 to 4 Petri dishes containing Hank's solution to eliminate contamination with blood. After rinsing, the extraneous tissue about the trachea (esophagus, connective tissue, etc.) was removed with sterile forceps. The trachea was then sectioned into thin rings approximately 1 mm in thickness with limited stretching to minimize trauma. Some 15 to 20 rings were obtained from each trachea. Trachea rings obtained in this fashion were finally transferred to 60 x 15 mm Falcon Petri dishes. Each dish contained 4 rings floating freely in 4 ml of the culture medium.

The medium consisted of Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 100 units/ml of penicillin, 100 \(\mu g/ml\) of streptomycin, 0.1 \(\mu g/ml\) of vitamin A, and 0.01 \(\mu g/ml\) of vitamin E. Initial trials with M199 and with Dulbecco's medium unsupplemented by vitamins A and E gave results inferior to the supplemented medium that was used.

All cultures were gassed with a mixture of 90% \(O_2\) and 10% \(CO_2\) for 20 minutes until the phenol red indicator showed a color equivalent to pH 7.2-7.4. Once the cultures were initiated, it was found necessary to change the medium and to gas them every other day. Five percent \(CO_2\) in air proved less satisfactory than this high oxygen-high \(CO_2\) mixture.

Addition of test compounds to this basic medium was achieved by dissolving them in Tween 60.

Cultures were grown in the presence or absence of test substances as follows: (a) medium alone, (b) 0.1 ml Tween 60/100 ml medium (c) 2.3 \(\mu g\) 3-methylcholanthrene/100 ml medium, (d) 2.3 \(\mu g\) of 1,2-benz(a)anthracene/100 ml medium.
The 1,2-benz(a)anthracene was intended to provide exposure to an analog of 3-methylcholanthrene which, if not entirely noncarcinogenic, was at best a very weak carcinogen (7).

Control and experimental cultures were carried over a six-week period with serial harvesting at 1, 2, and 4 days and 1, 2, 3, 4, 5, and 6 weeks. Harvested rings were fixed in buffered 10% formalin for a week. They were dehydrated, imbedded in paraffin in the usual manner, sectioned at 3 to 10 microns, and stained with hematoxylin and eosin.

RESULTS

In general, survival of the tissues of the rings was excellent (almost 100%) for the initial 4 weeks of culture. Upon removal from the animals, the normal pseudostratified epithelium rapidly degenerated except for residual islands of ciliated cells. For the most part, only the basal layer was left. This rapidly grew to encompass all surfaces of the free-floating ring in doughnut fashion. [In control cultures, ciliated cells were seen off the outside as well as on the inside of the rings. These could be seen to be actively beating prior to harvesting (Figs. 1, 2).] Initially, epithelial regeneration consisted of a flat, 1- or 2-cell layer. Progression of growth from this stage gave rise to an epithelium which varied significantly in form and cellular characteristics depending upon the presence or absence of test materials in the medium. Mural connective tissue remained viable. Fibroblasts did not overgrow. Their growth appeared inhibited by the epithelial coat. Apart from true inhibition, there was low grade proliferation of chondrocytes beneath the perichondrium at the tips of the cartilaginous rings. The encompassing epithelium may interfere with the diffusion of oxygen and nutrients into the deeper layers.

The tissue patterns and cellular characteristics of the epithelium of the variously exposed tracheal rings at 1, 2, 3, and 5 weeks are described below. No description is included for those cultures grown in medium to which only Tween 60 was added for these were found to be identical to the untreated cultures.

Control

1 Week. The epithelium regenerated to a cuboidal form superimposed on a basal layer. The newly formed cuboidal surface cells were generally free of cilia, but occasionally cilia were seen. Nuclei were fairly free of cilia, but occasionally cilia were seen. Nuclei were large and vesicular (Fig. 3a). 2 Weeks. There was imperfect pseudostratification with cells somewhat less elongated than in the original state. Newly formed cilia were prominent and actively beating on the entire internal and external surfaces of the rings. Basal cells were interposed between the elongated ciliated cells. The former had somewhat smaller and more deeply staining nuclei than the latter. In general, this was the stage most closely resembling normal respiratory epithelium (Fig. 3b).

3 Weeks. The epithelium regressed. Cilia were lost and a relatively static epithelium was present. This consisted of a 1- to 2-cell layer of flat cells with uniform vesicular nuclei (Fig. 3c).

5 Weeks. The static quality of the above-described 3-week stage was demonstrated by little evidence of further change at 5 weeks. Cells were flat to cuboidal, nuclei were uniform and fairly vesicular, and there was little evidence of proliferative activity (Fig. 3d).

3-Methylcholanthrene

1 Week. In contrast to the control cultures, the regenerating epithelium showed disorganization and much more marked nuclear variability in regard to size, shape, and staining properties. The cell to cell relationship appeared quite haphazard and the appearance of polarization to the basement membrane was lost (Fig. 4a).

2 Weeks. Proliferation was more active and epithelial thicknesses of 4-, 5-, and 6-cell layers were not unusual. There was loss of polarity of cells and beginning of crowding and nodular proliferation of basal cells. Nuclear variability was more marked, and the nuclear cytoplasmic ratio increased. Here and there intercellular bridges could be seen, and there was some sense of maturation to flatter surface cells in what may be termed an atypical squamous metaplastic epithelium (Fig. 4b).

3 Weeks. Definite "epidermatization" of the epithelium occurred. There was striking nodular downgrowth of basal cells. These had the appearance of irregular "rete-peg." Progression to surface squamous differentiation was noted, but disorganization and loss of normal cell to cell orientation was characteristic. Nuclei showed extreme degrees of variability, being for the most part hyperchromatic, pleomorphic, and enlarged (Fig. 4c).

5 Weeks. Loss of polarity was more marked, and orderly differentiation toward a squamous epithelium was absent. There was no stepwise maturation to surface squamous cells. Throughout the entire thickness of epidermis, there were pleomorphic polygonal cells with nuclei varying considerably in size, shape, and staining properties. Occasional pyknotic nuclei presaged degeneration (Fig. 4d).

1,2-Benz(a)anthracene

1 Week. As in the control section, the epithelium consisted of a double cell layer. There was less tendency for cuboidal surface differentiation and the plump and vesicular nuclei showed none of the variability seen in the 3-methylcholanthrene-treated rings (Fig. 5a).

2 Weeks. Epithelium consisted of 2 or 3 layers of cells with no cilia differentiation as in the control and no metaplasia of the type seen with 3-methylcholanthrene. There was, rather, the simple imposition of undifferentiated, polygonal cells with little evidence of proliferative activity. The junction between epithelium and connective tissue of the lamina propria was indistinct (Fig. 5b).

3 Weeks. A relatively static double-cell-layered epidermis was seen. Nuclei were large and vesicular, but tended to be fairly uniform. Some surface cells were flattened; others were cuboidal (Fig. 5c).

5 Weeks. Degeneration of surface layers of cells occurred with superficial groups showing pyknosis and karyorrhexis. Basal cells remained fairly static (Fig. 5d).

DISCUSSION

Survival of rat tracheal rings in liquid culture medium may be achieved with regularity for up to four weeks and, with lesser
frequency, up to six weeks. The technic of free-floating organ culture requires a thin section and a high oxygen atmosphere. These permit adequate oxygenation of all tissues. The standard tissue culture media with minor modifications are entirely suitable for the support of all tissue elements. The retention of architectural organization and the relatively balanced growth of tissues made this a most useful tool for the study of tissue to tissue interaction with and without exogenous manipulation.

This organ culture system was employed in the evaluation of the effects of 3-methylcholanthrene on the larger conducting airways of the respiratory system. The connective tissue and cartilage appeared unaffected by the carcinogen. It was obvious that epithelial growth was more florid in the 3-methylcholanthrene-treated cultures than in the untreated or 1,2-benz(a)anthracene-treated cultures.

Within one week, while the untreated cultures were re-differentiating toward a ciliated epithelium, the 3-methylcholanthrene-treated epithelium grew to produce an imperfectly oriented metaplastic squamous epithelium. By three weeks this latter epithelium showed nodular downgrowth of basal epithelium and markedly variable and atypical nuclei. At this time, the epithelium of the untreated cultures had regressed to a torpid, 1- to 2-cell layer, and only slightly more evidence of cellular activity was observed in the 1,2-benz(a)anthracene-treated cultures. There was little change in the untreated epithelium at 5 weeks; cell degeneration and death was first observed in the 1,2-benz(a)anthracene-treated cultures; the 3-methylcholanthrene-treated group of cultures continued to be characterized by a multilayered atypically oriented metaplastic epithelium with bizarre hyperchromatic nuclei.

No frank invasion through basement membrane and into underlying connective tissue was observed in cultures maintained for periods up to six weeks.

Attempts at in vitro chemical induction of cancers in cell and organ systems have met with limited success. Berwald and Sachs (2) have described malignant transformation of hamster embryo cells by polycyclic hydrocarbons. Exposure of cultures of these cells to 3,4-benzo(a)pyrene, 3-methylcholanthrene, and 7,12-dimethylbenz(a)anthracene produced cell lines which grew continuously in culture, demonstrated loss of contact inhibition, and produced tumors upon reimplantation into adult hamsters. The transformed cells were also shown to be more resistant to the cytotoxic effect of hydrocarbon carcinogens. Thus, the changes observed after exposure to chemical carcinogens paralleled those seen after infection with polyoma virus. Comparable effects were not obtained with mouse embryo cultures where transformation occurred in untreated cultures with the same frequency as that seen in treated cultures.

The problem introduced by the observation of "spontaneous" transformation in untreated (control) cultures was well pointed up by the earlier studies of Earle et al. (6), who emphasized that the changes in control and those in the carcinogen-treated cultures differed only in degree. A similar difficulty is encountered in the interpretation of the report by Borenfreund et al. (3). These authors have described the association of chromosomal abnormalities observed after carcinogen treatment of cultures of Chinese hamster lung tissue cells with the ability of treated cells to produce tumors upon inoculation into the Syrian hamster cheek pouch. These same changes were observed in untreated cultures which also gave rise to tumors on in vitro implantation, albeit rarely. Borenfreund and her associates emphasize the difficulty of distinguishing between the selective effects of carcinogen on spontaneously occurring transformations and the true induction of transformation by carcinogen. In light of the resistance of viral-transformed cells to the cytotoxic and cytoidal effects of chemical carcinogens (3, 5), the possibility of chemical carcinogens acting simply as a selective factor favoring the emergence of transformed cells cannot be dismissed.

The observation of serial changes in most, if not all, of the individual cells of an entire tissue in the form of a fairly uniform field effect, as described above in the organ cultures treated with carcinogen, appears to argue strongly for a direct action of carcinogen in the cellular alterations produced. These results confirm and extend the direct tissue effect described by Lasnitzki (12, 13) in a series of studies of the changes produced by 3,4-benzo(a)pyrene and tobacco tar condensate on cultures of human fetal lung. Varying degrees of hyperplasia with cellular and nuclear atypicity were produced. Similar changes were observed in organ cultures of adult mouse prostate exposed to 3-methylcholanthrene. Crocker et al. (4) cultured long intact tracheal segments of suckling rats on a solid plasma clot medium containing hydrocarbon carcinogens. Increased rates of mitosis, as measured by tritiated thymidine uptake, and hyperplasia and metaplasia were observed.

The significance of the changes observed in the tracheal ring cultures described in this report, as well as those produced by Lasnitzki (12, 13) and by Crocker et al. (4), still must be evaluated in terms of carcinogenesis. Studies bearing on this have been reported by Heidelberger and Tyte (8). These authors have used Lasnitzki's system to expose adult mouse prostate to 3-methylcholanthrene or 9,10-dimethyl-1,2-benz(a)anthracene. The cultures were exposed for one week, then grown in carcinogen-free medium for two more weeks. After dissociation of cells, permanent cell lines were evolved which, on injection, yielded tumors. The authors describe epithelial abnormalities in the intact organ cultures, but most of the tumors produced after dispersal were sarcoma. There was, then, little correlation between the epithelial changes and the predominant type of tumor produced. The possibility of some selective action of carcinogen on the highly unstable and tumor-prone mouse fibroblast (14) still exists.

The extensive and fairly uniform changes produced in the epithelium of the cultured tracheal rings closely parallel the changes observed by Auerbach et al. (1) in their study of human bronchial epithelium in cigarette smokers. These changes certainly accompany and probably precede the development of lung cancer. Whether they are truly stages in the expression of malignant transformation remains to be demonstrated. They are quite comparable to the changes seen in the course of the induction of bronchogenic carcinomas in rats by ionizing radiation as demonstrated by serial sacrifice (10). The organ culture technie may eventually permit the direct observation of the unquestionable transformation of normal epithelial cells to invasive malignant cells.

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ORGAN CULTURE OF RAT TRACHEA


Fig. 1. Intact tracheal ring after 2 weeks of culture in medium alone. The entire ring is invested by epithelium. The cartilage and connective tissue are well preserved. H & E, X 60.

Fig. 2. Detail of tracheal ring from Fig. 1. The control culture at 2 weeks demonstrates well-differentiated ciliated epithelium on the inner and outer surfaces of the sectioned ring. H & E, X 415.

Fig. 3. Epithelium in control culture (medium alone). H & E, X 1000. (a) 1 week. Nonciliated cuboidal cells over a basal layer of cells. Nuclei are uniform. (b) 2 weeks. Well-differentiated pseudodisclstratified ciliated cells approximating the appearance of intact respiratory epithelium. (c) 3 weeks. Loss of differentiation with epithelium reduced to a 1- to 2-cell layer. Nuclei are uniform. (d) 5 weeks. Little change from 3 weeks in the character of the 2-cell-layer epithelium with uniform nuclei.

Fig. 4. Epithelium in culture with 3-methylcholanthrene added to medium. H & E, X 1000. (a) 1 week. Disorganized regeneration with loss of polarity and nuclear variability. (b) 2 weeks. Active proliferation has produced 4-, 5-, and 6-cell-layered epithelium. Loss of polarity, beginning basal crowding, and marked nuclear variability in an imperfect squamous metaplastic epithelium are seen. (c) 3 weeks. Squamous metaplasia with basal nodular overgrowth. Nuclei show marked atypia. (d) 5 weeks. Loss of squamous differentiation. Atypical nuclei are seen throughout the thickness of the epithelium.

Fig. 5. Epithelium in culture with 1,2-benz(a)anthracene added. H & E, X H 1000. (a) 1 week. Double cell layer with uniform plump nuclei. The surface layer shows a less well-defined cuboidal character than that of the control culture but none of the disorganization seen in the 3-methylcholanthrene culture. (b) 2 weeks. Superimposition of 2 or 3 layers of undifferentiated polygonal cells. No cilia formation nor squamous metaplasia is seen. (c) 3 weeks. There is a static 2-cell-layered epidermis. Nuclei are large, vesicular, and fairly uniform. (d) 5 weeks. Surface layers show degenerative changes with pyknosis and karyorrhexis. There is little evidence of basal proliferation.

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