

# Quantitative Assessment of Generalized Epithelial Changes in Tracheal Mucosa following Exposure to 7,12-Dimethylbenz(a)anthracene<sup>1</sup>

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## ABSTRACT

Sequential morphological changes occurring after brief carcinogen exposures of heterotopic tracheal transplants in rats were semiquantitatively studied. Tracheas were exposed to 7,12-dimethylbenz(a)anthracene for 1, 2, or 4 weeks, during which time means of 138, 152, and 160  $\mu\text{g}$  7,12-dimethylbenz(a)anthracene, respectively, were delivered. The first two types of exposures resulted only in generalized epithelial changes; these included hyperplasia and early metaplasia, both of which regressed rapidly, and persistent atrophic alterations. No focal epithelial lesions or tumors developed. The third type of exposure (160  $\mu\text{g}$  7,12-dimethylbenz(a)anthracene delivered in 4 weeks) resulted in the appearance of generalized mucosal changes with long-lasting, severe inhibition of mucus production. In addition, focal metaplastic lesions reappeared at 4 to 8 months after exposure, and invasive carcinomas developed after 1 year with an incidence of 9%. Overall carcinoma incidence, including carcinoma *in situ*, was 15%.

The studies emphasize the importance of the duration of carcinogen exposure, and they demonstrate the emergence of focal lesions when effective carcinogenic exposures are being used. The possible significance of epithelial atrophy in the pathogenesis of cancer in this experimental model is discussed.

## INTRODUCTION

The trachea and bronchi are normally lined by pseudostratified mucociliary epithelium. However, a variety of morphological changes are frequently observed in the respiratory tracts of both humans and experimental animals. Such changes can often be linked to one or more of a spectrum of factors, including exposure to cigarette smoke (1, 12, 13, 21), chemical carcinogens (3, 10, 22), high-energy irradiation (14), mechanical irritation (5), infectious agents (18, 20), or vitamin A deficiency (10, 24). The changes described are hyperplastic or metaplastic in nature and frequently show either the lack of, or drastically altered, differentiation. Similar changes have been produced in respiratory tract epithelium in organ culture with chemical carcinogens (4, 19), asbestos (16), or vitamin A-deficient culture medium (15). When these morphological al-

terations occur *in vivo* following exposure to known carcinogenic agents or complex carcinogenic mixtures, such as cigarette smoke, the generally accepted opinion is that some of them must represent various stages, and possibly irreversible steps, in the development of cancer. However, with the information available in most studies, it is impossible to determine which of the observed pathological changes are due to acute cytotoxic effects and which are due to the carcinogenic process. The primary purpose of the studies described in this and the accompanying report (23) was to investigate the temporal sequence of epithelial changes occurring in the conducting airways subsequent to a brief carcinogenic insult. In this way, we hoped to avoid the confounding interference of acutely toxic effects that make it difficult to interpret most morphogenesis studies conducted on human or animal respiratory tract tissue. The principal approach chosen was to expose a limited segment of the airways of rats (tracheas) to a known quantity of carcinogen for a short period of time and to investigate quantitatively the epithelial changes occurring during the subsequent exposure-free interval. Rat tracheas grafted *s.c.* to isogenic recipients were used as target tissue for the carcinogen. A previous study (7) has shown that various epithelial lesions, similar in appearance to those observed in humans exposed to carcinogenic insults (1), can be produced in this tracheal transplant model. It was known from previous studies (8, 17) that the tumor incidence occurring in carcinogen-exposed transplants is dose dependent. However, at high carcinogen doses, there is a rapid conversion of the tracheal epithelium directly into massive dysplasia and invasive carcinoma. To avoid this problem, the dosage regimen which was used was selected in an attempt to obtain a tumor response characterized both by a long latent period and a tumor incidence of approximately 10%. In this manner, we hoped to expand the time scale of the progression of epithelial alterations, thereby allowing us to investigate the evolution, as well as the possible reversion, of those alterations. Two types of epithelial abnormalities were observed in our studies: "generalized" epithelial changes affecting large areas of tracheal mucosa; and localized focal lesions. The former are discussed here and the latter are described in the accompanying paper (23).

## MATERIALS AND METHODS

**Carcinogen Pellets.** DMBA<sup>4</sup> powder (Eastman Kodak Co., Rochester, N. Y.) was melted at 122° and mixed with melted laboratory grade beeswax (Fisher Scientific Co., Fairlawn, N. J.) so that the mixture contained 0.67% carcinogen by weight. Cylindrical pellets (14 x 155 mm) were formed by use of a

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<sup>4</sup> The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; PAS, periodic acid-Schiff; i.l., intraluminal.

stainless steel pellet maker as described previously (6). The pellets made in this manner weighed  $24.5 \pm 1.1$  (S.D.) mg and contained 165  $\mu\text{g}$  DMBA. The concentration of the carcinogen in the pellets was confirmed by dissolving the pellets in benzene and measuring the absorption of the resulting solution by UV spectrophotometry using a molar extinction coefficient of  $\epsilon_{301}$  (79,000). In this range, neither benzene nor beeswax interferes with the UV absorption of DMBA.

**Animals and Tracheal Transplants.** Tracheas from female Fischer F-344 rats were transplanted s.c. in the retroscapular region to isogenic recipients, 2 tracheas/animal. Four weeks posttransplantation, the tracheas are vascularized, are morphologically indistinguishable from host tracheas, and actively secrete mucus (6).

For insertion of carcinogen pellets, the animal was lightly anesthetized, and a small incision was made in the skin over the graft. The trachea was exposed, and a small cut was made near one end. The beeswax pellet containing the DMBA was inserted into the trachea through the incision. The trachea was then closed with silk suture, and the skin wound was closed with metal clips. Beeswax pellets without carcinogen were used as controls.

**Experimental Design.** A total of 126 tracheal transplants were used for the assessment of epithelial changes induced by carcinogen exposure. Forty-two transplants each were exposed for 1, 2, or 4 weeks. Exposure was terminated by removing pellets from the transplants. Six tracheas from each group were harvested for microscopic examination when the pellets were removed and at 1, 2, 4, 8, 16, and 32 weeks after termination of the carcinogen exposure. The carcinogen doses delivered during 1, 2, and 4 weeks of exposure were determined by quantitating the amount of carcinogen remaining in the pellets.

To relate our observations to tumor formation, groups of transplants were exposed to DMBA for 1, 2, or 4 weeks as described above. Animals were palpated biweekly, and tumors were removed when they were 2 cm in any dimension. All animals carrying exposed tracheal transplants were allowed to survive their full life span or until tumors developed in both grafts.

**Histopathological Assessment.** Histological changes in the tracheal epithelium were semiquantitatively assessed. Alterations in the pattern of epithelial differentiation were used to define 4 major groups of tissue change. The following epithelial states were scored: (a) squamous metaplasia, with or without keratinization; (b) hyperplasias, both columnar and transitional, the latter resembling bladder epithelium; (c) atrophic epithelium, often irregular and poorly differentiated, characterized by pleomorphism; and (d) normal- or near-normal-appearing epithelium, including the low cuboidal epithelium in control tracheas after 2 months. Tracheas were fixed in Bouin's solution, cross-sectioned into 1.5-mm rings, and embedded in paraffin. This procedure provided from 10 to 15 small cylindrical segments from each trachea. Three paraffin sections, 5  $\mu\text{m}$  thick, were cut from each block and were stained with hematoxylin and eosin.

The extent of each of the epithelial states in individual rings was estimated to the closest 10% of the tracheal circumference using a calibrated eyepiece. In each trachea, each cross-section was examined, and the percentage of tracheal circumference occupied by each of the morphological types was

estimated. From these values, the mean percentage of the tracheal surface occupied by each epithelial type was calculated for each trachea. The scores obtained for the 6 tracheas in each identically treated group were averaged to give an estimate of the preponderance of the various epithelial abnormalities present following each specified period of carcinogen exposure and recovery.

The persistence of the effects of previous carcinogen exposure was also evaluated by noting (a) the presence of keratinizing squamous metaplasia and (b) the absence of one measure of normal differentiation, that being the presence of mucus-producing cells. The percentage of histological sections containing keratinizing squamous metaplasia was determined for each trachea, and a mean percentage was obtained from the 6 tracheas in each group. Presence of mucus-producing cells was determined from sections stained with PAS reagent. Tracheas were rated according to the following scheme: 0, no detectable positive cells; +1, few or scattered positive cells; +2, large numbers of positive cells. A mean numerical rating for each time point was determined from the scores of the 6 tracheas in each group.

## RESULTS

**Epithelial Changes Occurring during Carcinogen Exposure.** The release of DMBA from i.l. pellets is summarized in Chart 1. It can be seen that nearly 140  $\mu\text{g}$  of the 165  $\mu\text{g}$  DMBA contained in the pellets at the start of exposure, *i.e.*, 85%, are delivered during the first week. During the subsequent 3 weeks, an additional 20  $\mu\text{g}$  of carcinogen are released. Chart 2 demonstrates the morphological response of the tracheal epithelium during the 4 weeks of carcinogen exposure. The various classes of epithelial morphology which were observed are illustrated in Figs. 1 to 5. At 1 week, roughly 40% of the mucosa is hyperplastic, and approximately 10% shows squamous metaplasia; the rest is hyperplastic, with little "normal" mucosa remaining, suggesting that the entire tracheal mucosa is exposed to the carcinogen. At 4 weeks of exposure, much of the squamous metaplasia has disappeared, about 30% of the tracheal lining is hyperplastic, 20% is atrophic, and the rest (30 to 35%) has returned to a near-normal state showing mostly cuboidal to low columnar epithelium with or without cilia.

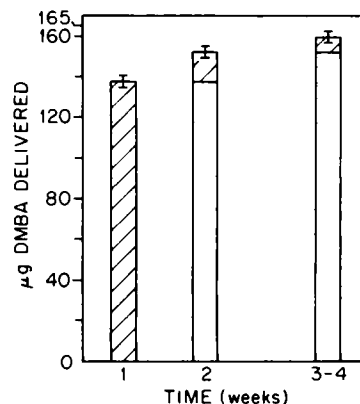
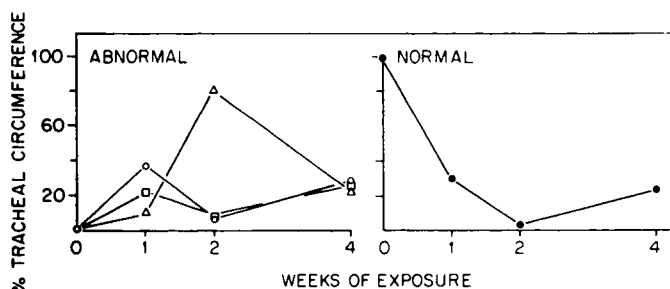


Chart 1. Delivery of carcinogen to tracheal transplants from beeswax pellets initially containing 165  $\mu\text{g}$  DMBA. Hatched columns, amount delivered during the given time; open columns, amount previously delivered; bars, S.D.

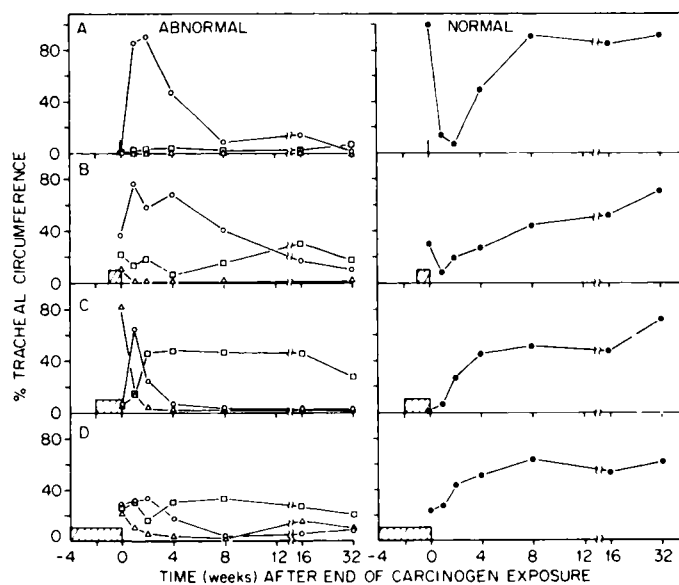


**Chart 2.** Epithelial response of tracheal transplants during exposure to beeswax pellets initially containing 165  $\mu\text{g}$  DMBA. The estimated percentage of tracheal surface area occupied by various epithelial morphologies is shown. ●, normal- or near-normal-appearing epithelium;  $\Delta$ , squamous metaplasia;  $\circ$ , hyperplasias;  $\square$ , atrophic, pleomorphic, and irregular cuboidal epithelium. Values are based on the mean of 6 tracheas per time point.

**Generalized Epithelial Changes Occurring after Carcinogen Exposure.** We will describe here those morphological changes which occupy large areas of the tracheal lining. The focal lesions which develop on this general background will be discussed in the accompanying paper (23). Chart 3 summarizes the changes in epithelial morphology occurring after the carcinogenic insult is discontinued. Tracheas exposed to DMBA for only 1 week show widespread hyperplasia for at least 2 months. Squamous metaplasia is observed only during the first postexposure week. Between 10 and 30% of the tracheal surface is covered by atrophic epithelium throughout the study. By 2 months, nearly 50% of the epithelial lining has returned to a near-normal state.

In tracheas exposed for 2 weeks, more than 80% of the tracheal surface is lined by squamous epithelium which disappears rather rapidly within 2 weeks after cessation of exposure, giving way to a transient hyperplasia. The hyperplastic phase is followed by an atrophic phase which persists for many months. About half of the epithelial surface is lined by atrophic epithelium; the other half or more is covered with near-normal epithelium from about 1 month on. In tracheas exposed for 4 weeks (only 5 to 10  $\mu\text{g}$  of carcinogen were released between 2 and 4 weeks), roughly equal amounts of surface area were classified as hyperplastic, metaplastic, atrophic, or normal. The amount of hyperplastic and metaplastic epithelium declines during the subsequent 2 months. At 4 months, a significant amount of squamous metaplasia has reappeared. Atrophy is a prominent feature throughout. Nevertheless, approximately 60% of the epithelium has returned to the near-normal state by 2 months. In tracheas receiving "empty" beeswax pellets, small atrophic patches of epithelium were occasionally seen. These were not counted, however, since they always covered far less than 10% of the luminal surface of the tracheas. These small lesions also did not show the pleomorphic features of the atrophies in the carcinogen-exposed tracheas.

The same histological material was also evaluated in a different manner, by scoring cross-sections for PAS-positive cells or for keratin-producing epithelium. The results in principle confirm the findings reported above (Table 1). With 1 week of DMBA exposure, evidence of PAS-positive cells is found in most cross-sections at 2 months and appears similar to that for unexposed controls. After exposure for 2 weeks, recovery of PAS-positive cells appears to be slower and less complete. After 4 weeks of DMBA exposure, very little restoration of PAS-positive cells seems to occur, suggesting that the epithelium is



**Chart 3.** Epithelial changes observed after cessation of carcinogen exposure. Extent of epithelial changes induced by exposure for 1, 2, or 4 weeks to pellets initially containing 165  $\mu\text{g}$  DMBA. The estimated percentage of tracheal surface area occupied by various epithelial morphologies is shown. ●, normal- or near-normal-appearing epithelium;  $\Delta$ , squamous metaplasia;  $\circ$ , hyperplasias;  $\square$ , atrophic, pleomorphic, and irregular cuboidal epithelium. Values are based on the mean of 6 tracheas per time point. A, Beeswax vehicle only; B, 1 week exposure; C, 2 weeks exposure; D, 4 weeks exposure. Hatched columns, the duration of carcinogen exposure.

functionally more severely damaged than would have been predicted from the histological appearance of the epithelial lining (Chart 3). Scoring of the sections containing epithelium actively producing keratin showed essentially the reverse picture. With 1 week of exposure, no keratin was found except at 0 time, *i.e.*, at the end of exposure. With a DMBA exposure of 2 weeks, a high percentage of sections contained keratin at 0 time and 1 week. Thereafter, keratin-producing epithelium was only found in rare instances. With 4 weeks of exposure, there was a prolonged decline in the percentage of areas producing keratin between 1 and 8 weeks of recovery, followed by a marked increase at 4 and 8 months (most of this increase is due to focal lesions).<sup>5</sup> Thus, with exposure to DMBA extended through 4 weeks, long-lasting disturbance of normal epithelial differentiation is evident long after the acutely toxic effects have disappeared.

**Tumor Incidence in DMBA-exposed Tracheas.** Groups of tracheas exposed to DMBA for 1, 2, or 4 weeks were observed for up to 27 months to determine tumor incidence. No tumors developed in a total of 44 tracheas exposed for 1 or 2 weeks which had received an average of 138 or 152  $\mu\text{g}$  of DMBA, respectively. In contrast, a 9% invasive carcinoma incidence was observed in 86 tracheas exposed to DMBA for 4 weeks (delivered dose, 160  $\mu\text{g}$ ). All of the tumors were squamous cell carcinomas. The mean tumor induction time was 14 months.

## DISCUSSION

The purpose of the studies described in the present and the accompanying paper (23) is to analyze sequential morphological changes which occur as a result of a brief carcinogen

<sup>5</sup> D. C. Topping and P. Nettesheim, unpublished observations.

Table 1  
Evidence for mucus or keratin production in tracheas following DMBA exposure

| Exposure to DMBA (wk) | PAS-positive <sup>a</sup> material at the following times |         |        |         |         |         |         | Keratin associated with squamous epithelium at the following times |        |        |        |       |        |         |
|-----------------------|---|---------|--------|---------|---------|---------|---------|--|--------|--------|--------|-------|--------|---------|
|                       | 0 wk  | 1 wk    | 2 wk   | 4 wk    | 8 wk    | 16 wk   | 32 wk   | 0 wk   | 1 wk   | 2 wk   | 4 wk   | 8 wk  | 16 wk  | 32 wk   |
| 0 <sup>b</sup>        | +2 <sup>c</sup> (6) <sup>d</sup>                          | +2 (6)  | +2 (6) | +2 (6)  | +2 (6)  | +2 (6)  | +2 (6)  | 0  | 0      | 0      | 0      | 0     | 0      | 0       |
| 1                     | 0   | <+1 (1) | 0      | <+1 (1) | 1-2 (5) | 1-2 (5) | +2 (6)  | 13 ± 11 <sup>e</sup>   | 0      | 0      | 0      | 0     | 0      | 0       |
| 2                     | 0   | 0       | 0      | <+1 (2) | 1-2 (5) | 1-2 (3) | 1-2 (5) | 77 ± 16  | 66 ± 8 | 2 ± 2  | 0      | 2 ± 2 | 5 ± 5  | 0       |
| 4                     | 0   | 0       | 0      | <+1 (2) | <+1 (1) | <+1 (1) | <+1 (1) | 39 ± 13  | 18 ± 7 | 16 ± 5 | 11 ± 4 | 4 ± 2 | 23 ± 9 | 22 ± 14 |

<sup>a</sup> PAS-positive cells were quantitated as follows: 0, none; +1, few or scattered positive cells; +2, many positive cells.

<sup>b</sup> Determined from tracheas implanted with blank beeswax pellets. Zero time point represents resting, unexposed transplants.

<sup>c</sup> Predominant response within each group.

<sup>d</sup> Numbers in parentheses, number of tracheas (of 6) with active secretion.

<sup>e</sup> Mean percentage of tracheal segments with epithelium producing keratin ± S.D. ( $n \pm 6$  tracheas).

exposure in respiratory tract epithelium without the confounding complications of continuous or repetitive exposure. It is hoped this analysis will lead to a better understanding of the morphogenesis of neoplasia and, in particular, of the significance of specific epithelial abnormalities in the conducting airways.

The tracheal transplant system seemed particularly well suited for this type of investigation because only a relatively small segment of respiratory tract mucosa had to be surveyed morphologically, and, therefore, the types of mucosal abnormalities could be categorized and quantitatively assessed. Dose-response studies conducted previously (8) had shown that high tumor incidences with short induction times occurred at doses of DMBA above 300 µg. At such carcinogen dose levels, distinctly separate morphological lesions, as described in humans (1), were not observed. Rather, the development of the acute hyperplastic-metaplastic reactions merged with that of the dysplastic-neoplastic reactions. In the present studies, we chose a considerably lower carcinogen dose, which produced a tumor incidence of 9%. This frequency is comparable to that observed in high-risk groups of heavy smokers (9). Since the tumor latency was long, we had the opportunity to conduct the major part of the investigation after the acute toxic effects had subsided and to study the appearance and possible reversal of epithelial lesions over many months.

Our data clearly demonstrate that a massive and widespread hyperplastic-metaplastic response developing early during carcinogen exposure is not necessarily an indication of neoplastic transformation, since it occurred in tracheas exposed for only 2 weeks, an exposure which does not result in tumor formation. In fact, the morphological changes induced by this brief carcinogen exposure, even though drastic and widespread, are almost completely reversible (Chart 3; Table 1) and are probably primarily a manifestation of the acute toxicity associated with DMBA. It is tempting to speculate that the reversal of mucosal alterations in the bronchi of exsmokers, as reported by Auerbach *et al.* (2), may be a similar or related phenomenon. This does not necessarily mean, however, that some "carcinogen-altered" cells do not persist within the recovering epithelium.

Three findings stand out from the present study as noteworthy: (a) marked differences in response in spite of only minor differences in dose; (b) varied nature and focality of lesions in spite of uniform exposure; and (c) long-term persistence of epithelial damage. Major differences were apparent in the early as well as the late response of the tracheal epithelium to the carcinogen in spite of only minor differences in dose. These were, however, coupled with significant differences in exposure

duration. One week of exposure was characterized by early widespread hyperplasia, and 2 weeks of exposure were characterized by early widespread metaplasia. These changes were almost completely reversible in both instances. Neither exposure was tumorigenic. Four weeks of exposure were characterized by an early, mostly reversible metaplasia and, most significantly, the reappearance of focal metaplastic lesions and development of tumors at late time points. The late focal metaplasias [described in detail in the accompanying paper (23)] which appeared in this study on a background of either near-normal or atrophic epithelium 2 to 4 months after cessation of carcinogen exposure were detected in significant numbers only in the group of tracheas receiving a tumorigenic exposure.

Of special interest is the fact that in spite of the apparent uniformity of exposure, as judged by the uniformity of the early hyperplastic-metaplastic response involving most of the epithelial surface, focal metaplastic and atypical lesions arise 4 and 8 months after carcinogen exposure in the group receiving an effective tumorigenic exposure. This "focality" of the late response indicates either that the nature of the damage sustained by the exposed epithelial cell population is not uniform or that the damage is not expressed morphologically in a uniform manner.

An interesting observation made in the present study is the persistence of the atrophic changes estimated to cover 30 to 40% of the mucosal surface in most of the carcinogen-exposed tracheas. This morphological category encompasses a variety of changes characterized by undifferentiated and "pleomorphic" epithelia (*i.e.*, irregular cell sizes and shapes), all of which are, however, characterized by marked atrophy. These late atrophies are an indication of persisting damage of the cell renewal system and are a reminder that metaplastic-dysplastic lesions are not the only "late effects" of carcinogen exposure. The possibility exists that some pathogenetic relationship exists between this atrophy and the emergence of neoplasia. Such a connection has been reported for some types of gastric cancers (11). Conceivably, the chronically damaged, growth-inhibited tissue provides a "permissive" environment in which the few initiated cells can effectively compete and ultimately obtain a growth advantage over the remainder of the cells.

Another important observation was made in the course of these studies, which, although not directly related to the morphological aspects of this investigation, will be discussed at some length. Three different exposure durations were used in this experiment. Doses of 138 and 152 µg of DMBA delivered over 1 and 2 weeks, respectively, did not produce tumors. The

Table 2  
Percentage of carcinoma incidence in tracheas<sup>a</sup> exposed for various lengths of time to DMBA

| 12 wk           |             | 4 wk            |                     | 1 to 2 wk        |             |
|-----------------|-------------|-----------------|---------------------|------------------|-------------|
| Dose ( $\mu$ g) | % of tumors | Dose ( $\mu$ g) | % of tumors         | Dose ( $\mu$ g)  | % of tumors |
| 100             | 43          | 97              | 18                  | 138 <sup>b</sup> | 0           |
| 150             | 44          | 160             | 15, 19 <sup>c</sup> | 152 <sup>d</sup> | 0           |
| 165             | 46          | 190             | 20 <sup>c</sup>     | 500 <sup>d</sup> | 0           |
| 200             | 55          |                 |                     |                  |             |

<sup>a</sup> Twenty to 80 tracheas/group.

<sup>b</sup> Amount delivered in 1 week.

<sup>c</sup> Removal of DMBA-containing pellet at 4 weeks followed by insertion of blank beeswax pellet.

<sup>d</sup> Amount delivered in 2 weeks.

third group of tracheas was exposed for an additional 2 weeks, during which time only an additional 8  $\mu$ g of DMBA was delivered. However, the tumor study showed an incidence of 9% invasive and 6% noninvasive carcinomas in this group. It should also be remembered that significant numbers of late-appearing focal lesions appeared only following 4 weeks of carcinogen exposure. We know from previous experiments that 10  $\mu$ g of DMBA is not a carcinogenic dose in this system (8).

Because of the potential significance of this finding, we reviewed the tumor-incidence data from a series of independent studies recently completed in our laboratory (Table 2) in which tracheas were exposed to doses of DMBA ranging from 100 to 500  $\mu$ g. The exposure durations ranged from 1 week to 3 months. Exposure periods of 4 weeks or shorter were terminated by removal of the carcinogen-containing pellet and, in some cases, by replacing them with blank beeswax pellets. The data show that a range of doses of DMBA delivered over 1 to 2 weeks are nontumorigenic, whereas tumors appear at incidences of 15 to 20% following 4 weeks exposure and of 40 to 50% following prolonged exposure. The difference does not seem to be related to the physical presence of beeswax pellets, since no significant difference in tumor incidence was observed in groups of tracheas reimplanted with blank pellets made of the vehicle alone.

Obviously, the decisive factor determining tumor incidence in this dose range is not total carcinogen dose but rather the duration of exposure. Apparently, only  $\mu$ g quantities of carcinogen are required during the latter part of exposure to dramatically increase the tumor response.

Our interpretation of this finding is that the first, relatively brief carcinogen exposure, while not sufficient to induce tumors, results in carcinogen-altered cells, which through continuing exposure are further altered and ultimately give rise to tumors. This second phase of the process may be a promotion-like effect and is brought about by exposures which are by themselves neither tumorigenic nor significantly cytotoxic as far as can be determined by histological means.

The carcinogen-altered cells do not form focal metaplastic lesions such as are observed following tumorigenic exposures. It is conceivable, however, that some of the generalized epithelial alterations which were observed in the present study from 2 to 32 weeks after nontumorigenic exposures to DMBA are morphological manifestations of the first phase of carcinogenesis and may, therefore, represent crucial intermediates in the

morphogenesis of cancer.

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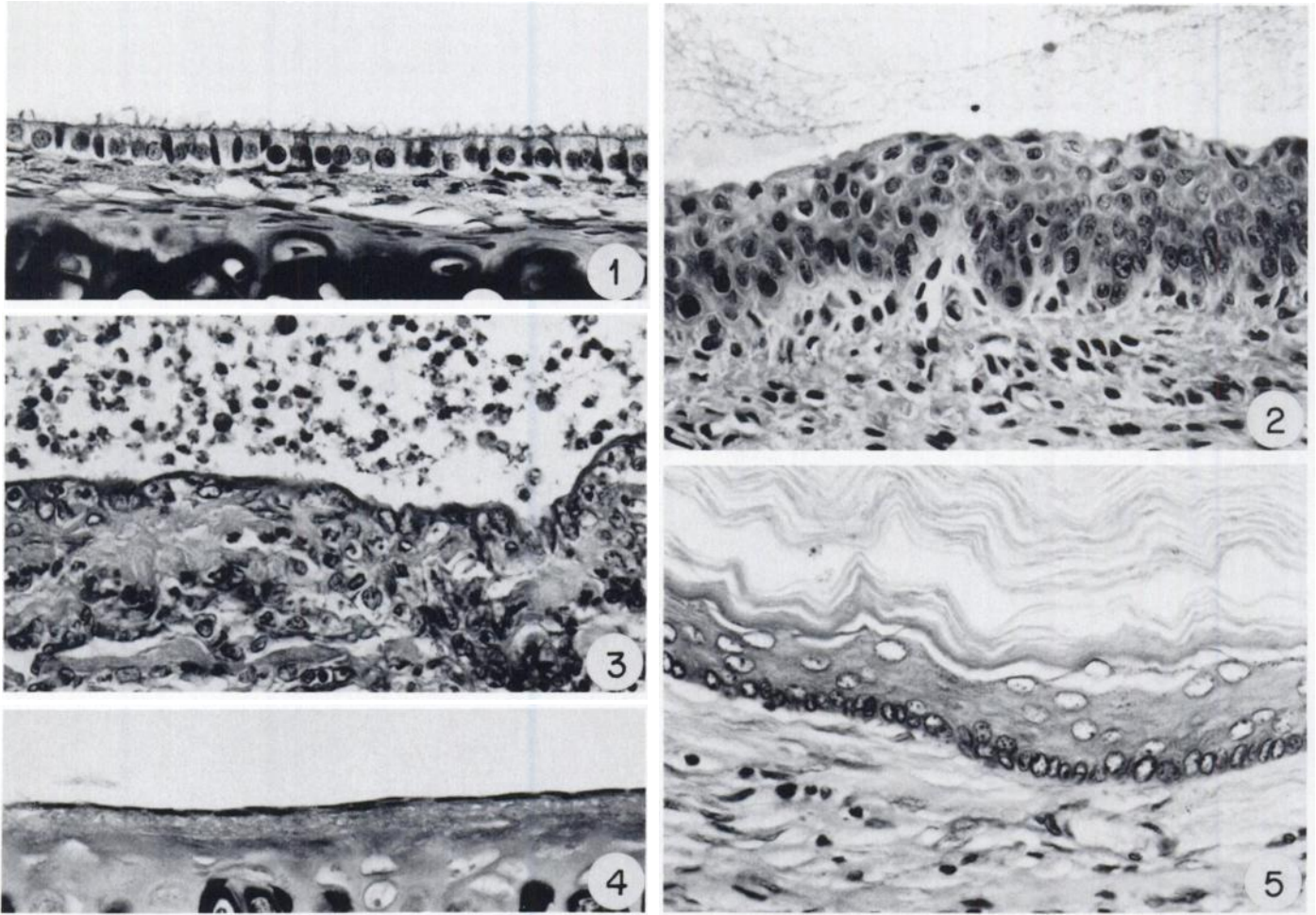


Fig. 1. Normal-appearing epithelium in established tracheal transplant. H & E,  $\times 420$ .  
Fig. 2. Basal or transitional cell hyperplasia, 1-week exposure to DMBA. H & E,  $\times 420$ .  
Fig. 3. Disorganized pleomorphic atrophy, 2-week recovery from 4-week exposure to DMBA. H & E,  $\times 420$ .  
Fig. 4. Severely attenuated atrophic epithelium, 16-week recovery from 2-week exposure to DMBA. H & E,  $\times 420$ .  
Fig. 5. Uniformly keratinizing squamous metaplasia, 2-week exposure to DMBA. H & E,  $\times 420$ .