Pathologic Changes Induced in Respiratory Tract Mucosa by Polycyclic Hydrocarbons of Differing Carcinogenic Activity

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Seven aromatic polycyclic hydrocarbons (PCHs) were investigated for their toxic effects on respiratory mucosa: benzo(*e*)pyrene (BeP), pyrene, anthracene, benz(*a*)anthracene (BaA), dibenz(*a,c*)anthracene (DBacA), benzo (*a*)pyrene (BaP), and dimethylbenz(*a*)anthracene (DMBA). The compounds were chosen because they comprise a spectrum of PCHs ranging from noncarcinogens, to initiators, to weak and strong carcinogens. All of them except DMBA are environmentally relevant chemicals. The chemicals were tested over an 8-week period. Heterotopic tracheal transplants were continuously exposed and the histopathologic effects induced by the various PCHs were periodically assessed semiquantitatively. All PCHs exhibited varying degrees of toxicity for respiratory epithelium and submucosa. BeP clearly showed the least toxicity followed by pyrene and anthracene. BaA and DBacA caused marked epithelial and submucosal changes. In addition to epithelial hyperplasia, undifferentiated epithelium and squamous metaplasia developed. Marked mononuclear infiltration occurred in the subepithelial connective tissue. With BaP the epithelial and submucosal changes were similar but were much stronger. DMBA was the most toxic substance, causing epithelial necrosis followed by generalized keratinizing squamous metaplasia; the subepithelial changes consisted of an early acellular exudate and, later (at 8 weeks), marked condensation and hyalinization of the lamina propria. The toxic response pattern of the tracheal mucosa to carcinogenic agents was characterized by the chronicity of epithelial and connective tissue damage, as opposed to the short-lived hyperplastic and inflammatory response elicited by the noncarcinogens and weak initiators. (Am J Pathol 93:311–324, 1978)

CARCINOGENIC POLYCYCLIC HYDROCARBONS (PCHs) are suspected to be the most important etiologic agents involved in the pathogenesis of lung cancer, the most common source being tobacco smoke.[1] They are also believed to play a role as occupational [2,3] and possibly as general environmental carcinogens,[4–6] although the latter is still a matter of debate. (See References 7 and 8 for reviews on this subject). Most

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studies concerned with the effect of PCHs on the airways focus on the carcinogenicity of this class of compounds. The cytotoxicity of several of the PCHs is well documented [9,10] and appears to be due, at least in part, to metabolic products such as phenols.[11] However, a systematic and quantitative assessment of the pathogenicity of PCHs with varying degrees of carcinogenicity for respiratory tract tissues has never been attempted.

The purpose of the present study was to investigate a number of environmentally relevant PCHs for their toxic effects on respiratory tract mucosa. A semiquantitative method was used to assess the degree of damage to the epithelium and the subepithelial connective tissue. The chemicals examined were pyrene, benzo(*e*)pyrene (BeP), anthracene, benz(*a*)anthracene (BaA), and dibenz(*a,c*)anthracene (DBacA), and two extensively studied carcinogenic PCHs, ie, benzo(*a*)pyrene (BaP) and dimethylbenz(*a*)anthracene (DMBA).

The tracheal transplant model was used for these investigations since it permits exposure of a defined segment of respiratory tract to a known quantity of test substance.[12] Heterotopic tracheal grafts are maintained under the dorsal skin of recipient rats isogenic to the donors. The grafts are exposed to the test substance from an intraluminal beeswax pellet which releases the material slowly over a period of several weeks in a pattern similar to that observed with subcutaneous PCH deposits in lipid solvents.[13] The mucosal changes resulting from the toxicity of the various PCHs were monitored and semiquantitatively assessed over an exposure period of 8 weeks (except for pyrene, for which the exposure time was 2 weeks).

The experiments showed that all of the tested PCHs, both noncarcinogenic and carcinogenic, produce toxic changes in the tracheal mucosa, although to varying degrees. The response pattern of the tracheal mucosa roughly parallels the initiator–carcinogen activity of the compounds.

Materials and Methods

Chemicals

Pyrene, BaP, BeP, and DBacA were obtained from Aldrich Chemical Company (Milwaukee, Wis.); BaA and DMBA were obtained from Eastman Kodak Co. (Rochester, N. Y.); and anthracene was obtained from Matheson, Coleman & Bell (Norwood, Ohio). All of the PCHs were recrystallized before use. Beeswax (laboratory grade, Fisher Scientific Co., Fairlawn, N. J.) was heated to melting and vacuum-filtered through a steam-jacketed funnel with a medium-porosity fritted glass disk (pore diameter, 10 to 15 μ). The PCHs were dissolved in beeswax in a concentration of 4.2% by weight. Cylindric pellets (14 × 1.5 mm) were formed by use of a stainless steel pellet mold as described previously.[12] Pellets made in this way each contained 1 mg of the test substance. The concentration of

compound in the pellets was determined by ultraviolet spectrophotometry. The following molar extinction coefficients were used for determining the concentrations of the different PCHs in benzene: BaP, ϵ_{368} (80960); BeP, ϵ_{334} (35600); anthracene, ϵ_{378} (8065); pyrene, ϵ_{336} (46775); DBacA, ϵ_{289} (122990); BaA, ϵ_{282} (98610); DMBA, ϵ_{301} (79000). In this range neither beeswax nor benzene interferes with the ultraviolet absorption of any of the test substances.

In Vitro Release of Compounds Into Fetal Calf Serum

The release of each compound from beeswax pellets was measured by use of fetal calf serum as a suspending medium. Pellets were placed individually into small Erlenmeyer flasks containing 6.0 ml of sterile heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.). The flasks were covered with foil and agitated in a water bath at 37 C at 100 cycles/min. The serum was changed daily. At the end of 1 week, the pellets were removed, rinsed, and assayed as described above.

Animals and Tissue Preparation

Tracheas from Fischer F-344 male rats were transplanted subcutaneously in the post-scapular region to isogenic recipients, two tracheas per animal. Four weeks later they were exposed to PCHs. At this time the tracheas are fully vascularized and established, are morphologically normal, and secrete mucus. For implantation of pellets, the animals were lightly anesthetized and a small incision was made in the skin over the graft. The trachea was exposed, a small cut was made near one end, and a beeswax pellet containing the test substance was inserted into the trachea through the incision. The trachea was closed with a suture, and the skin wound was closed with metal clips.

For each test chemical, 30 transplanted tracheas were used. Six grafts each were removed at 3 days and at 1, 2, 4, and 8 weeks after insertion of the pellets and were assayed for remaining PCH. Tissues were fixed for histologic assessment in Bouin's fluid. Tracheas were cross-sectioned into 1.5-mm–thick rings and embedded in paraffin. This procedure provided 10 to 15 tracheal cross-sections from each trachea. Paraffin sections (4 μ thick) were cut and stained with hematoxylin and eosin (H&E). Selected tissues were also stained with alcian blue, periodic acid–Schiff (PAS), and Masson trichrome.

Histologic Assessment

Histologic changes in tracheal epithelium and subepithelial tissues were semi-quantitatively assessed. The following epithelial abnormalities were scored: a) columnar hyperplasia; b) undifferentiated epithelium (this includes so-called transitional hyperplasia[14] as well as low cuboidal and pleomorphic epithelium); c) squamous metaplasia; and d) atrophy and erosion.

The *extent* of each epithelial change, ie, the proportion of tracheal circumference occupied by each of the epithelial types listed above, was assessed as follows. For each trachea, every other cross-section was examined, and the percent tracheal circumference showing morphologic changes *a* through *d* was estimated (any particular morphologic change recorded may occupy as little as 10% and as much as 100% of any one cross-section). The mean percent cross-sectional area occupied by each of the morphologic changes was calculated for each trachea. The scores obtained for the six tracheas per group were averaged to give an estimate of the preponderance of the various epithelial abnormalities induced by exposure to PCH. Untreated tracheal transplants established for 3 to 4 months were used as references to establish a base line. The *intensity* of each noted epithelial change was graded as mild, moderate, or severe. For hyperplastic, transitional, and metaplastic responses, the thickness of the epithelial lining provided the criterion for assessment of intensity. Severe responses were characterized by over five layers of cells,

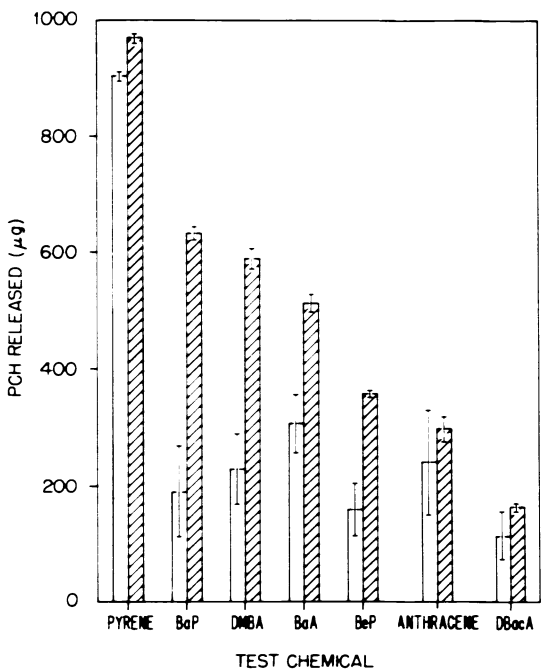
moderate responses by a thickness of three to five rows of cells, and mild responses by an average thickness of two to three cells. The mild hyperplasia category also included areas of marked cellular hypertrophy. Erosion and atrophy occurred to a significant extent only in DMBA-treated grafts and will not be shown.

Subepithelial changes were assessed as follows: Three broad categories were recognized: a) edema and/or granulocytic infiltration; b) histiocytic–fibroblastic response usually associated with mononuclear cell infiltration; and c) fibrosis and hyalinization. Each trachea was classified according to the presence or absence of these changes, which were graded on a 0 to 3+ scale. The values assigned each categorical change reflected both estimated extent as well as intensity of the change. Gradations of intensity were determined as follows: 0, similar to untreated tracheal grafts; +1, noticeable reaction, but without disturbance of the normal architecture of the lamina propria; +2, focal moderate disturbance of normal architecture caused by infiltration of cellular elements or fluid; +3, severe and generalized alteration of subepithelial elements caused by extensive infiltration of cellular elements or edematous fluid. The mean score for each categorical change for each group and time point was arrived at by adding the individual scores and dividing them by the number of tracheas examined.

Results

PCH Release From Pellets

To interpret the pathologic effects of the various carcinogenic and noncarcinogenic PCHs on respiratory tract mucosa, it was essential to

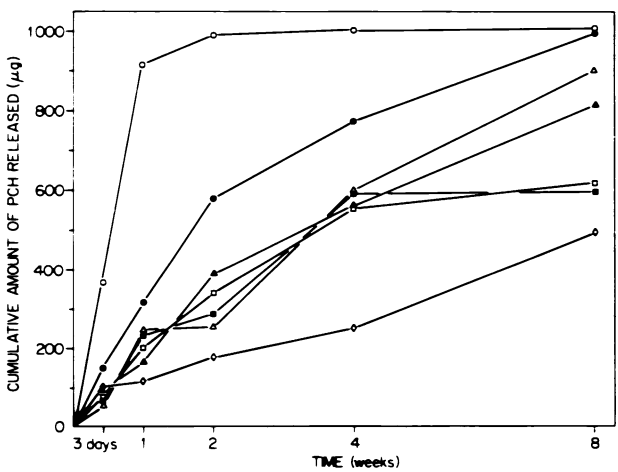


TEXT-FIGURE 1—*In vitro* release of PCHs from beeswax pellets. *Cross-hatched columns*, cumulative release at 1 week *in vitro*; *open columns*, *in vivo* release at 1 week, given for comparison (data from experiment summarized in Text-figure 2). Each bar represents the mean amount of PCH released ± SE from four to six pellets. The ratios of *in vitro* to *in vivo* release are as follows: pyrene, 1.1; BaP, 3.3; DMBA, 2.6; BaA, 1.7; BeP, 2.2; anthracene, 1.2; DBacA, 1.4.

have some information on the release rates of the various compounds from the pellets used for delivery of the test substances. An *in vitro* study was carried out to learn the effect of the physicochemical properties of the test substances on release without interference from biologic variables. The second release experiment was carried out as part of the pathologic study (see below) to measure release rates *in vivo* during exposure of the tracheal grafts.

Text-figure 1 summarizes the results of the *in vitro* experiment. The PCHs are arranged in descending order according to the amount released within 1 week. (The 1-week *in vivo* release values are given for comparison.) Nearly 100% of the pyrene was released into the serum within 1 week, compared with only 15% of the DBacA. The other PCHs fell between these two extremes, with 30 to 60% delivered within 1 week.

Text-figure 2 summarizes the *in vivo* release of the various PCHs from pellets inserted into tracheal grafts over a 2-month period. As in the *in vitro* study, pyrene was released most rapidly and DBacA most slowly. The other compounds show intermediate rates which do not necessarily coincide with those suggested by the *in vitro* data. (See also Text-figure 1.) In most cases, 50 to 60% of the test substance had been delivered by 4 weeks. The data indicate that the tracheal tissues are continuously exposed to the PCHs for the duration of the study, except for the group receiving pyrene. The ratios of *in vitro* to *in vivo* release (Text-figure 1) indicate that except for pyrene, anthracene, and DBacA, *in vivo* release is much slower than release *in vitro*. Also, the variability *in vivo* is much greater than *in vitro*.

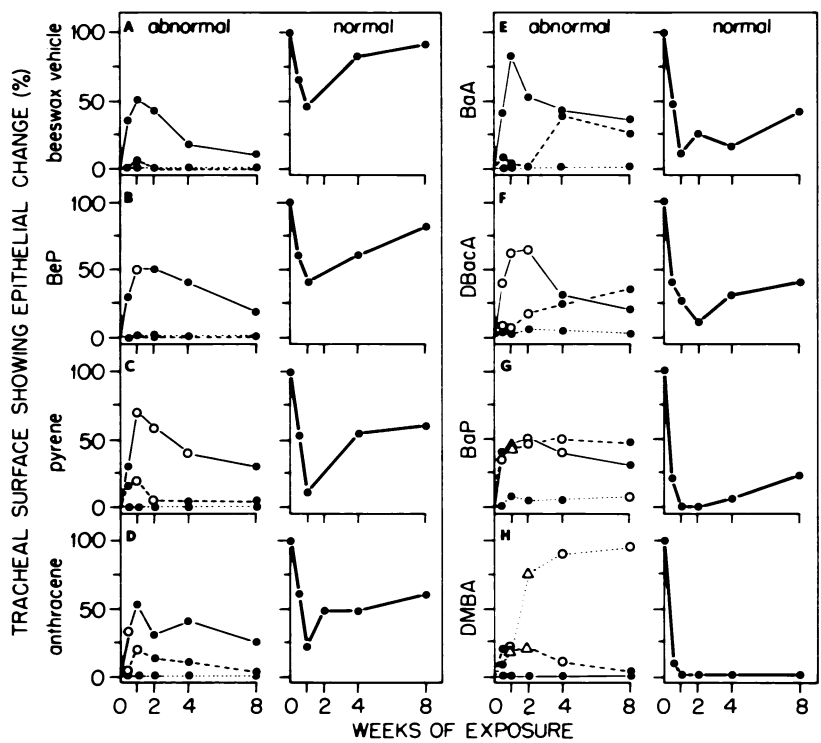


TEXT-FIGURE 2—*In vivo* release of PCHs in tracheal transplants. Pellets were removed at intervals over an 8-week period to determine the amount of PCH released. All pellets contained 1 mg of PCH at the start of the experiment. △, anthracene; ▲, BeP; □, BaP; ■, DMBA; ●, BaA; ◇, DBacA; ○, pyrene. Each point represents the mean of six pellets.

Histopathologic Effects of PCHs

The effect of the various PCHs on the tracheal epithelium and subepithelial tissue was determined semiquantitatively over a period of 8 weeks.

The epithelial changes will be described first, then the alterations of the subepithelial connective tissue. The epithelium of the resting established transplant is low columnar and mucociliary in character (Figure 1A). Insertion of beeswax pellets produced a generalized, but mild and short-lived, hyperplasia (Figure 1B and Text-figure 3A). By 4 weeks the epithelial lining was essentially normal except for an occasional small focus of "transitional" epithelium. The epithelial changes induced by BeP were only slightly greater than those seen after the beeswax vehicle alone. The main difference was a more intense hyperplastic response at 1 week,



TEXT-FIGURE 3—Extent and intensity of epithelial changes induced by various PCHs. The estimated percent tracheal surface area occupied by various epithelial morphologies is shown. *Thick lines*, normal-appearing epithelium; *thin lines*, hyperplasia; *dashed lines*, transitional epithelium; *dotted lines*, squamous metaplasia. Values are based on six tracheas per time point. (For details on the assessment of epithelial change, see *Materials and Methods*.) Each trachea received 1 mg of PCH incorporated in beeswax. Control tracheas received pellets made of beeswax only. The intensity of the epithelial change is indicated by the following: ●, mild; ○, moderate; △, severe.

which involved approximately half the tracheal surface (Text-figure 3B). Pyrene, anthracene, and BaA (Text-figures 3C through E) caused not only a more long-lasting hyperplastic response but also, at least in the case of pyrene, a more severe mucociliary hyperplasia (Figure 1C). A notable characteristic of this response was a marked goblet cell hyperplasia (Figure 1D). Probably more important is the appearance of undifferentiated epithelium, some of it in the form of so-called transitional hyperplasia [14] which we consider to be a more severe toxic response because of the loss of normal differentiation (Figure 1E). The right graph for each substance in Text-figure 3, which summarizes the return of the epithelium to normal, shows that even at 8 weeks following initial exposure to pyrene, anthracene, and BaA, only half of the tracheal surface area had become normal (Text-figures 3C through E), indicating that these three PCHs have a significant, long-lasting toxic effect. With DBacA, toxicity was even more pronounced (Text-figure 3F). Hyperplasia was strong and widespread. The amount of transitional epithelium continued to increase over the 8-week observation period. A considerable portion of the transitional epithelium produced by this chemical was irregular and "pleomorphic" (Figure 1F), one to three cell layers thick, and almost completely undifferentiated. In addition, small but measurable areas of squamous metaplasia developed. At 8 weeks, approximately 60% of the epithelium was still abnormal.

The two carcinogenic PCHs, BaP and DMBA, caused an epithelial response markedly different from that of the other five PCHs (Text-figures 3G and H). BaP induced a severe and long-lasting hyperplasia and transitional hyperplasia. In addition, there were metaplasias, mostly focal, some of which became quite thick. They were nonkeratinizing in most cases. At 8 weeks, 75% of the epithelium continued to be abnormal (including some atrophic changes [not shown]). The effect of DMBA was even more dramatic. This PCH was so toxic that approximately 30% of the epithelium initially became necrotic (not shown). From Day 7 on, almost the entire tracheal surface rapidly turned into an often keratinizing, severely hyperplastic squamous metaplasia (Figure 1G), and at 8 weeks many foci with cellular atypia were seen, as was extensive epithelial downgrowth (Figure 1H). The entire epithelial surface appeared severely abnormal at 8 weeks.

The subepithelial connective tissue changes are summarized in Table 1. Some acute inflammation, ie, edema and/or granulocyte infiltration, occurred with all PCHs, but it was particularly strong with DMBA. With this carcinogen, a severe, almost completely acellular edema occurred (Figure 2A). A subacute inflammation characterized by mononuclear

Table 1—Submucosal Response to PCHs*

Response	Weeks of exposure	Compound							
		Beeswax	BeP	Pyrene	Anthracene	BaA	DBacA	BaP	DMBA
Edema + granulocytic infiltrate†	0.5	+	−	+	+	+	+	+	++
	1	−	+	+	+	−	−	+	+++
	2	−	+	+	+	−	+	+	+++
	4	−	−	−	+	++	+	+	++
	8	−	−	−	−	+	+	+	+
Mononuclear infiltrate	0.5	+	−	+	+	+	+	+	+
	1	+	+	+	+	++	+	++	−
	2	−	+	+	++	++	++	++	+
	4	−	+	+	++	+++	++	+++	+
	8	−	−	+	+	++	+++	+++	+
Fibrosis + hyalinization	0.5	−	−	−	−	−	−	−	−
	1	−	−	−	−	−	−	+	−
	2	−	−	−	−	+	−	++	−
	4	−	−	−	−	++	++	++	−
	8	−	−	−	+	+	++	++	+++

* Severity of submucosal responses at each time point by exposure to pellets containing 1 mg of test PCH. See text for details on assessment procedures.

† With all chemicals except DMBA, there was both edema and granulocyte infiltration. In the case of DMBA, there was only marked edema.

+, mild response; ++, moderate response; +++, severe response; −, no response or similar to untreated grafts.

infiltration and an increase in fibroblasts (Figure 2B) also was noticeable with all PCHs but was strongest with BaP, DBacA, BaA, and anthracene, in that order. Lymphocyte infiltration (Figure 2C) was striking with BaP. It was minor with BeP and pyrene as well as with DMBA. In the latter case the acellular exudate was the predominant feature during at least 4 of the 8 weeks of the study.

In the second half of the experiment, a considerable fibrosis and hyalinization developed in the lamina propria. This was by far strongest with DMBA (Figure 2D) but also occurred to a significant extent with BaP, DBacA, and BaA.

In summary, BeP and pyrene caused only a mild subepithelial inflammatory response (which was, however, noticeably greater than that observed with plain beeswax pellets). Anthracene, BaA, and DBacA caused a major toxic reaction of the mesenchymal subepithelial tissues; this reaction was even greater with the two carcinogenic PCHs.

Discussion

The tracheal transplant model appeared to us to be particularly well suited to assess the extent and degree of tissue damage induced in respiratory tract mucosa by a variety of noncarcinogenic and carcinogenic PCHs. The fact that sustained release of a known quantity of test chemicals in a standardized segment of trachea can be achieved makes the system useful for at least semiquantitative evaluation of the morphologic manifestations of toxicity. Several important insights pertinent to the still rather new methodology were gained by the comparative *in vitro* and *in vivo* release studies (Figure 1). *In vitro* release of PCHs was in most cases not predictive of *in vivo* release; it tended to be mare rapid, suggesting that the exchange of materials from the tracheal lumen to the circulation is comparatively slow. In the *in vitro* studies, release showed little variation. In contrast, *in vivo* variability was quite high. Most likely this is due to the changes induced by the chemicals in the tracheal wall, which in turn influence fluid exchange and release rates. We recently showed that this variability can be substantially reduced by slowing release rates from the pellets by modification of pellet matrix.[15]

For our studies we chose a series of PCHs with graded carcinogenic activity: DMBA and BaP, two strong carcinogens; DBacA, a weak carcinogen; BeP, a noncarcinogenic isomer of BaP; BaA, pyrene, and anthracene, considered by most to be noncarcinogens. The latter four compounds have been shown, however, to have different degrees of initiator activity (incomplete carcinogens), pyrene and anthracene being least

active in this regard. (For relevant information see References 16 through 20.)

The main questions we attempted to answer with our study are as follows: a) Are all of the seven PCHs, including the noncarcinogenic ones, toxic for respiratory tract mucosa? and b) Is there any relationship between the degree of carcinogenicity and the degree or type of toxicity as manifested by histopathologic changes? All seven PCHs showed measurable toxicity for epithelial as well as submucosal tissues. However, major differences in the degree of toxicity were noticed, which generally coincided with the degree of carcinogenicity. BeP, pyrene, and anthracene produced only a short-lived epithelial and submucosal response. For the most part, these compounds have been shown to be very weak or inactive as carcinogens. However, there is some discrepancy concerning the ability of BeP to act as an initiator. Van Duuren and co-workers [17] found that BeP was a poor initiator and an inactive carcinogen, while Scribner [19] found significant initiator activity with BeP. The discrepancy between these studies may be related to the considerably larger initiator dose used by Scribner. BaA, DBacA, BaP, and DMBA, in that order, show increasingly severe and long-lasting epithelial and submucosal pathologic changes. Only the latter three show significant squamous metaplasia and submucosal fibrosis. BaA does not fit into this pattern of correlation of toxicity and carcinogenicity since it is not considered to be a complete carcinogen; however, BaA is a fairly strong initiator.[16,18] This may explain its toxic response pattern, which is similar to that of DBacA. BeP and pyrene have been shown to be cocarcinogenic,[20] an activity which does not seem to be associated with the chronic toxicity seen with carcinogens. In this connection, it may be of interest that we recently found that the promoting agent TPA also causes a severe acute hyperplastic and inflammatory response in tracheal mucosa. This response is short-lived and recovery begins soon after the start of exposure.[21] The most striking feature of the response of tracheal tissues to carcinogens, which seems to distinguish it from the response to noncarcinogens, is the chronicity of the tissue damage. The tissue reaction to the noncarcinogens is mostly short-lived, although exposure continues. We are not implying that the carcinogen-induced morphologic changes described here are specific for the process of carcinogenesis (although some part of the overall tissue response probably is); rather, our data suggest that at least the chemicals studied here, besides causing neoplastic transformation, also bring about other profound and long-lasting tissue alterations. What makes the study of neoplastic development so difficult is the fact that carcinogens are obviously broad-spectrum cell and tissue toxins causing a variety of acute *and*

chronic cellular disorders, only some of which are related to the process of carcinogenesis.

In summary, our data show that a variety of PCHs, including those generally regarded as noncarcinogens (although some of them have initiating activity), cause significant epithelial and mesenchymal pathologic changes in respiratory tract mucosa. The noncarcinogenic agents trigger a toxic response pattern different from that of the carcinogens in that it is short-lived. Carcinogens, in contrast, produce responses which correspond in severity to their relative carcinogenicity. These characteristics are long-lasting tissue changes with persistent alterations of epithelial differentiation (metaplastic changes). The toxic tissue response patterns observed show a gradation roughly correspondent to the irritant, initiator, and carcinogenic activities of the chemicals.

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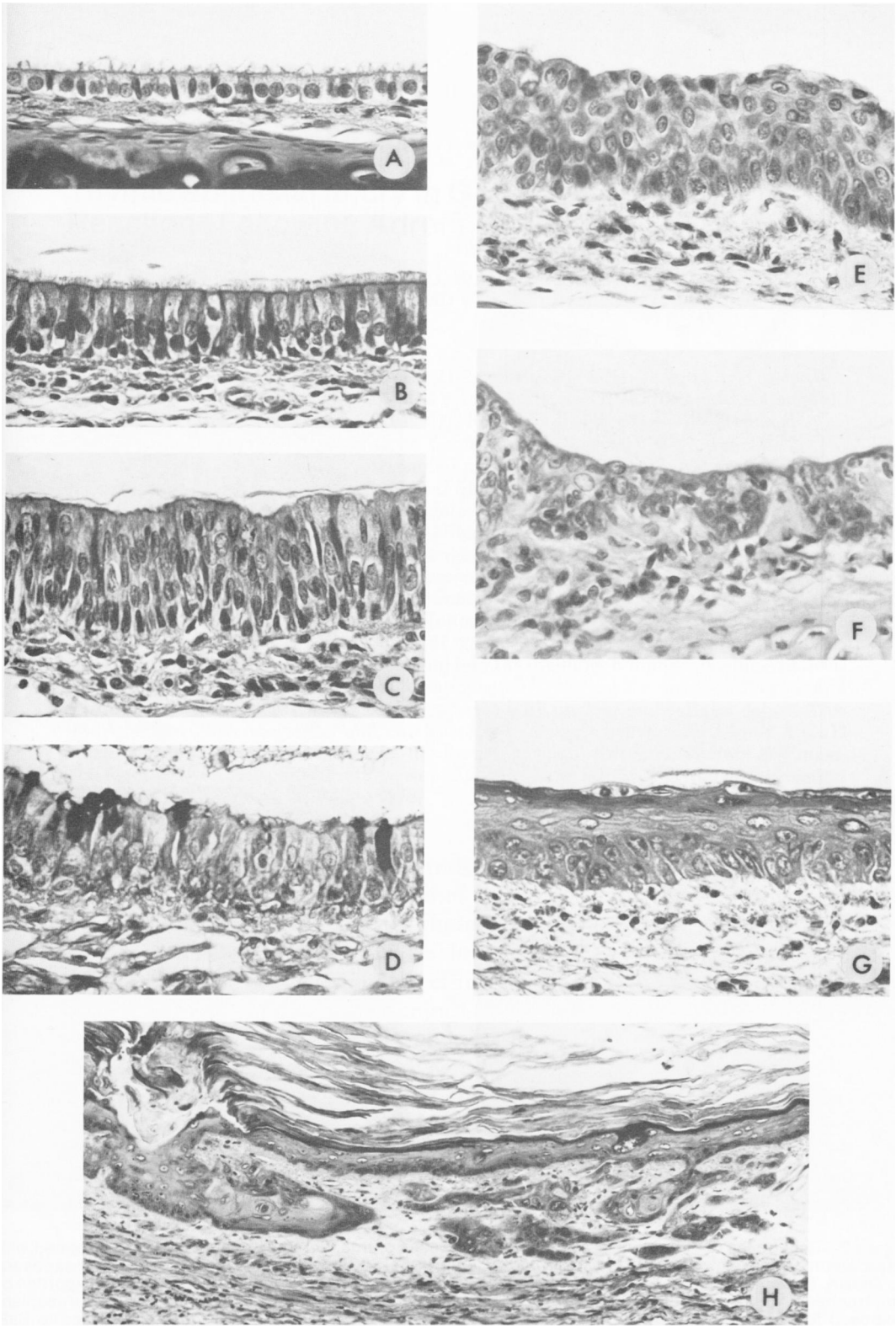

Figure 1—Epithelial responses to PCHs. All stained with H&E except where indicated otherwise. **A**—Normal mucociliary epithelium found in established tracheal transplants. (× 350) **B**—Mild mucociliary hyperplasia caused by the beeswax vehicle alone at 1 week of exposure. (× 350) **C**—Severe mucociliary hyperplasia resulting from 1 week of exposure to beeswax pellets containing 1 mg pyrene (× 350). **D**—Goblet cell hyperplasia; same exposure as in C. (PAS, × 350) **E**—Transitional hyperplasia after 1 week of exposure to pellets containing 1 mg DBacA. (× 350) **F**—"Pleomorphic" transitional epithelium occurring after 8 weeks of exposure to pellets containing 1 mg DBacA. (PAS, × 350) **G**—Squamous metaplasia occurring after 1 week of exposure to pellets containing 1 mg DMBA. (× 350) **H**—Heavily keratinizing squamous metaplasia occurring after 8 weeks of exposure to pellets containing 1 mg DMBA. Epithelial downgrowth and possible "micro-invasion" of neoplastic epithelium. (Masson trichrome, × 140)

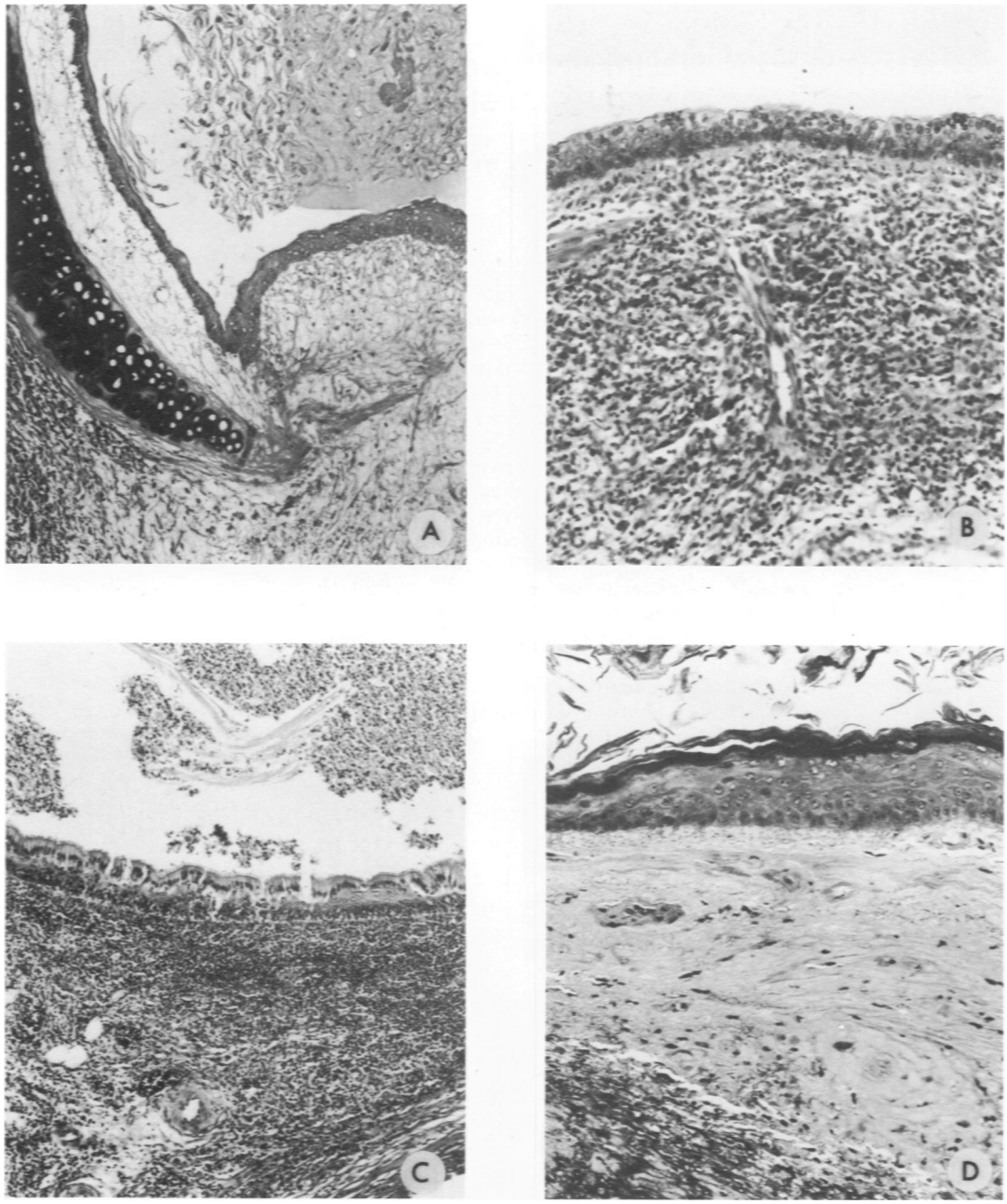

Figure 2—Responses of subepithelial and adventitial tissues of tracheal transplants to PCHs. All stained with Masson trichrome, except where indicated otherwise. **A**—Severe edema caused by 2 weeks of exposure to DMBA. Subepithelial areas are nearly acellular, both over the cartilage and in the membranous portion of the trachea. (× 88) **B**—Histiocytic-lymphocytic infiltrate in subepithelial connective tissue of transplant exposed to BaA for 2 weeks. (× 140) **C**—Massive lymphocytic infiltrate in transplant exposed to BaP for 4 weeks. Note hyperplastic basal cell layer and small patch of squamous metaplasia in the epithelium. (H&E, × 88) **D**—Condensation and hyalinization of the lamina propria following 8 weeks of exposure to DMBA. Note the heavily keratinizing metaplastic epithelium lining the lumenal surface. (× 140)