A Method for the Experimental Induction of Bronchogenic Carcinoma

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SUMMARY

A methodology for the experimental induction of bronchogenic carcinoma in conditions related to those of human exposure to respiratory carcinogens is described. Animal of choice is the hamster. Polynuclear hydrocarbon carcinogens are prepared as suspensions of fine crystalline particles and attached to a finely particulated inert dust which acts as a carrier; the mixed dust is suspended in saline and administered by intratracheal instillation. Penetration and distribution patterns are described. No necrosis and no chronic inflammatory reactions other than phagocytosis are induced. Repeated intratracheal instillations of benzo[a]pyrene with hematite dust induce up to 100% incidences of respiratory tract tumors, mostly bronchogenic carcinomas. The morphology of these tumors appears very close to that of human lung cancer; squamous cell carcinomas are the most frequent type followed by anaplastic carcinomas and a few adenocarcinomas. The experimental model presented here appears adequate for studies on factors involved in the pathogenesis of cancer of the lung.

INTRODUCTION

Cancer of the lung in man, particularly bronchogenic carcinoma, showed the most remarkable increase in incidence in the past 50 years and has become one of the most serious problems in public health and a great challenge in preventive medicine. Its origin has been linked with a number of environmental factors, notably inhalation of cigarette smoke and pollution of the air by chemicals and by radioactivity. Experimental study of the conditions of exposure to respiratory carcinogens is essential in order to gain some knowledge of the interplay of factors necessary to the pathogenesis of this form of cancer (42). Unfortunately, the search for an adequate method of experimental reproduction of bronchogenic carcinoma in animals, comparable to human lung cancer, met for a long time with a considerable lack of success. The earliest experiments that showed positive reproducible results were those with thread transfixions (1, 22, 52), those using intrabronchial pellets (22), and those using topical application of radioactive materials (2, 27, 28).

The experiments of Della Porta et al. (4), in our laboratory, had shown that the Syrian golden hamster was susceptible to the induction of tracheobronchial tumors, and that it was particularly suitable for lung experiments since untreated hamsters almost never develop spontaneous lung tumors and are quite resistant to pulmonary infections. However, the tumor incidence induced by their technic was very low and only one true bronchogenic carcinoma was induced; repetitions of some of their experiments with 7,12-dimethylbenz[a]anthracene (unpublished observation), and with benzo[a]pyrene (BP) (42), at the same dose levels, gave negative results. Further tests with the same technic by Gross et al. (12) only induced a few lung tumors, mostly peripheral, together with chronic pneumonitis. Besides, the carcinogen 7,12-dimethylbenz[a]anthracene, which is considerably cytotoxic, produced necrosis and broncho pneumonitis with areas of carnification.

Pylev (31–34) and Shabad (49, 50) reported positive results for the induction of bronchogenic carcinomas, following the intratracheal injection in rats of carcinogenic polycyclic hydrocarbons dispersed in a 4% casein suspension in balanced saline to which India-ink powder was added. The induced tumors were described as bronchogenic and were preceded by the finding of atypical hyperplasia and squamous metaplasia of the bronchial epithelium. Unfortunately, the rats were also found to develop chronic inflammatory changes and appreciable bronchiectases, as well as foci of interstitial fibrosis at the site of ink deposits (49). Herrold and Dunham (15) administered suspensions of BP in Tween 60 solutions to small groups of Syrian golden hamsters by intratracheal instillation. One group of 6 hamsters also received repeated urethan administrations. Six out of a total of 12 treated hamsters had tumors of the respiratory tract, including bronchogenic carcinomas. This technic has been subsequently used also by other investigators and its efficacy confirmed (29). As we noted before in discussing the conditions of exposure of the lung to carcinogens (42), the use

1 Supported by Grant No. E-214 from the American Cancer Society and by Research Project Contract No. MC-6 from the American Petroleum Institute. Parts of this work were presented at the 54th and 55th Annual Meetings of the American Association for Cancer Research held respectively in Toronto, Ont., May, 1963 and in Chicago, Ill., April, 1964; part of the iconography was shown in an exhibit at the Third Quadrennial Internat. Conference on Cancer, "Lung Tumors in Animals," Univ. of Perugia, Italy, June, 1965.

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Received February 16, 1967; accepted September 25, 1967.
of Tweens makes the conditions of tests further removed from those of human exposure, and somewhat complicated by the fact that Tween 60 has been found to have both carcinogenic and promoting activities for mouse skin (6).

Most of the positive experiments described above involved severe damage to lung tissues with extensive chronic inflammation and promoting activities for mouse skin (6). Those of human exposure, and somewhat complicated by the carcinogenesis. On the other hand, was somewhat difficult to accept, being contradictory to the experience acquired in most other types of carcinogenesis.

An alternative explanation for the failure of the experiments in which carcinogens were administered by more "physiologic" ways and the success of the few drastic techniques motivated our approach: that the essential factor involved was the adequate penetration of the carcinogen into lung tissues to attain a sufficient effective dose at the target site. Once the carcinogens penetrate, as through areas of necrosis, they can then affect the bronchial mucosa, while it appears that in methods in which the penetration of the carcinogen is impaired by the mucous and ciliary barriers, no effect could be exerted on the target tissue. Accepting this view as a working hypothesis, a method was required for the administration of carcinogens that would permit extensive penetration of the carcinogen into the lung tissues and yet avoid necrosis and chronic inflammation; this mode of administration would also have to be as closely related as possible to the conditions of human exposure to inhaled carcinogens.

Previous experience with the histogenesis of lung lesions due to various kinds of dusts had acquainted us with the penetration, distribution, and activity of dusts in the lung of experimental animals (30, 36, 37, 47, 48). From these data and others in the literature (3, 13, 14, 23, 25, 26), it became apparent that inert dusts of adequate particle size, such as carbon, iron oxide, and others, penetrate after inhalation or intratracheal injection in much the same way, through the walls of respiratory bronchioles and adjacent alveoli where they are subsequently phagocytized by macrophages and stored mostly in clusters of dust-laden macrophages without any further development of the cellular reactions.

The basic approach followed in our studies is to attach the carcinogen (a polynuclear hydrocarbon) to particles of an inert dust so that the dust can act as a carrier and transport the carcinogen through the bronchiolar and alveolar wall into the lung tissues. Once penetrated, the carcinogen is gradually dissolved and eluted out by interstitial fluids and allowed to reach the bronchial mucosa from the basal membrane side, thus circumventing the mucous and ciliary barrier. In doing so, no necrosis and no extensive chronic inflammation need be produced; this permits an experimental evaluation of their role, if any, in lung carcinogenesis. The methodologic approach proved successful and has been extensively used in our laboratory. We have presented an outline of the method and of some results in some earlier reports (38–42, 44, 45); this paper is devoted to a detailed description of the methodology and of the induced lesions in the first experimental group in which a high yield of respiratory tract tumors was induced.

MATERIALS AND METHODS

Preparation of Materials. The following materials were used: (a) BP (3,4-Benzopyrene, S.A.F. Hoffmann-La Roche & Co. Ltd., Basle, Switzerland), which was used as supplied since this compound, from the same source, had been found to be of such high purity that nothing would have been gained by attempting further purification; (b) hematite (ferric oxide, Fe₂O₃, Fisher Scientific Co., Fair Lawn, N. J.), as a fine dust of the following particle size distribution, determined by air settlement and microscopic examination: 99.9%, <10.0 μ; 99.7%, <7.5 μ; 98.9%, <5.0 μ; 98.2%, <4.0 μ; 97.9%, <3.0 μ; 97.1%, <2.0 μ; 94.1%, <1.0 μ; 77.7%, <0.5 μ; 47.8%, <0.25 μ; and (c) saline solution (0.9% sodium chloride, sterile, nonpyrogenic, Baxter Laboratories, Inc., Morton Grove, Ill.).

Equal weights of BP and hematite were transferred to a mullite mortar where they were mixed and then ground together for about one half hour, yielding a fine, homogeneously distributed dust containing 50% BP and 50% hematite by weight. The resulting dust appears microscopically composed of evenly distributed particles of both compounds attached together to form small aggregates. In preparing the mixed dust, care was taken to ensure that both samples were perfectly dry in order to avoid formation of larger clumps. The microscopic appearance of the mixed dust can best be appreciated using a microscope with attachments for tungsten light, polarized light, and UV fluorescence; in this way, the homogeneity of the dust and the relative distribution of the two components can be controlled, observing the red birefringence of hematite as well as the white-yellowish birefringence and the intense fluorescence of the carcinogen (Fig. 1). Following prolonged grinding, the particles of carcinogen and those of the carrier dust adhere together by surface adhesion, and the mixed dust can be suspended in saline without separating. If allowed to settle, it precipitates completely, showing that individual particles of carcinogen still adhere to those of hematite in suspension. The dust was stored in a glass bottle at room temperature in the dark. An adequate amount of dust was weighed out before each treatment and placed into a sterile 25-ml flask to which the saline solution was added to make a final concentration of 30 mg of dust per ml of saline. The suspension was shaken on a vibrator to ensure good dispersion (in later experiments it was found that a better dispersion is obtained by means of an ultrasonic probe) and then kept homogeneous by stirring on a magnetic stirrer during the whole duration of the experimental administration procedure.

Preparation of the mixed dusts could not be achieved just by adsorption since only microgram quantities of BP could be adsorbed on 1 gm of hematite, an amount too small for effective administration.

The dust was selected with the following criteria. It had to be an "inert" dust, not inducing fibrosis or any other marked tissue reaction besides phagocytosis and storage; its particle size had to be below the range that is optimal for complete penetration through the bronchiolar and alveolar walls and retention on the lung (approximately 1–3 microns) because this range is attained when the carcinogen particles are attached to the surface of the particles of carrier dust forming aggregates.
Hematite had been used in many previous studies and found to be particularly adequate as an inert material (48).

The carcinogen selected was BP because of its very low general and topic toxicity and because of its occurrence in human exposures.

**Selection of Animal Species.** Syrian golden hamsters of both sexes, obtained from the random bred colony of this Institute, were used. They were divided by sex at weaning and distributed six per cage in plastic cages with sterilized granular cellulose bedding in an air conditioned room. They were fed Rockland Rat Diet (A. E. Staley Mfg. Co., Decatur, Ill.) in pellets and tap water ad libitum.

The Syrian golden hamster was selected as the most appropriate experimental species after a series of trials and in consideration of the following facts. It has practically no spontaneous lung tumors: 1 instance of a pulmonary adenomatoid lesion was found years ago in over 500 controls in our laboratory (51, 55). No other spontaneous lung tumors have been seen in any other large group in our colony. On the other hand, hamsters had proved susceptible to the induction of tracheobronchial carcinomas by polycyclic hydrocarbons in the experiments of Della Porta et al. (4), a finding confirmed by the results obtained by others (12, 15, 29), and by those obtained in our laboratory using the present method, as well as by studies using remotely acting carcinogens such as diethylnitrosamine (7, 16). Furthermore, the hamster offers great resistance to chronic inflammatory conditions of the lung which are so common in other species such as rats and guinea pigs. It becomes thus possible to perform lung carcinogenesis studies in the absence of such conditions as well as studies especially designed for evaluating the role of purposely induced chronic inflammation in lung carcinogenesis. In addition, a large experience had been gathered in this laboratory on the carcinogenic response of the Syrian golden hamster to a variety of treatments (5, 11, 35, 43, 51, 53–55).

**Treatment.** The mixed dusts were administered in saline suspension by intratracheal instillation. We selected administration by intratracheal instillation rather than by inhalation mainly because the amount of dusts administered can be much more accurately quantitated, the treatment can be completed in a much shorter period of time, and the selected dose of dust can be entirely injected into the respiratory tract, while treatment in inhalation chambers exposes the whole animal to the dust with possible penetration through the skin and the gastrointestinal tract. The basic patterns of penetration and distribution of the dusts in the lungs are similar by either route of administration, and since saline is rapidly adsorbed in the tissues, the effects of the dust on the cellular reaction are essentially the same.

Before each treatment the animals were anesthetized with a dose of 0.4 ml of a 1% solution of Brevaltal Sodium (Sodium a-dl-1-methyl-5-allyl-5-(1-methyl-2-pentyl)barbituritate, Eli Lilly & Co., Indianapolis, Ind.) injected intraperitoneally through the abdominal wall previously disinfected with a few drops of 70% ethanol. This quickly acting barbiturate was selected in order to avoid the edema and other changes in the lungs often associated with ether anesthesia, and to permit rapid resumption of the normal respiratory activity. As soon as an animal was anesthetized, it was placed hanging on a slanted board, its back on the board and its mouth kept open by hanging the lower incisor teeth on a wire hook while the upper incisors were retained by a tight rubber band, as shown in Fig. 2. Then the selected volume of suspension was drawn out of the flask (while this was being stirred) using an 0.25-ml tuberulin syringe fitted with a blunt 19-gauge needle about 60 mm long and bent at a 135° angle at about 45 mm from the tip. A direct focusing headlight, worn by the operator, provided a clear view of the pharynx after the tongue of the hamster was gently pulled outward and laterally with a forceps. When needed, the oral cavity was cleaned of mucus with a small cotton swab. The blunt tip of the needle was then inserted under the epiglottis to uncover the vocal cords, and then lightly pushed between these into the tracheal lumen; a very light but definite bumping against the tracheal cartilage rings advises the operator that the needle is properly inserted. The needle was pushed almost to the bottom of the trachea, the suspension gently injected, and the needle withdrawn. Inspection of the pharynx was continued for a short time and the hamster was kept on the board for a minute or two to make sure no suspension regurgitated. Following the injection, the animals showed a brief apnea, after which they rapidly resumed regular respiration. With technical help for the anesthesia, each treatment only takes less than one minute. The apparatus used in preparing and injecting the suspensions was previously sterilized.

The hamsters were checked and weighed once weekly; animals in poor conditions were isolated in separate cages. The animals were either allowed to die spontaneously or were killed when moribund, and they were all autopsied.

A special technic was used for removing the lungs at autopsy in order to avoid their collapse and to fix them while fully expanded. The animals to be sacrificed were anesthetized as for treatment. The skin and muscle layers of the anterior neck region were dissected and the trachea exposed; a thread was inserted and loosely tied around the trachea; then the animal was bled by cutting the aorta through an opening in the abdominal wall; the ligature around the trachea was drawn tight and the chest cavity opened by cutting the diaphragm and the ribs on both sides and removing the sternum while carefully avoiding damage to the lungs. The lungs, still fully expanded, were excised en bloc with the trachea and mediastinal organs by grabbing the distal end of the esophagus with a forceps and pulling upwards while dissecting the loose connective tissue. A small lead weight was then attached to the tracheal ligature and the whole block immersed in 10% neutral buffered formalin. Such method of fixation allows an excellent view of the pulmonary architecture, and at the same time it avoids artifacts due to forced introduction of fixative into the bronchial tree (24). Following fixation, the pulmonary lobes were separated and sectioned each following the axis of the main bronchus. The trachea with the stem bronchi were also processed. Dehydration in alcohol was carried out under vacuum until all air was removed from the specimens; paraffin sections were prepared from all lobes as well as the trachea, the regional lymph nodes, and any other pertinent tissues.

Group 1 consisted of 30♂ and 30♀, 11 weeks old at the
beginning of the experiment; they received a course of 15 intratracheal instillations given once weekly, each instillation consisting of 6 mg of mixed dust (3 mg BP and 3 mg hematite) in 0.2 ml saline. Group 2 consisted of 24 δ and 24 ♀ in the same experimental conditions as Group 1; they received 15 intratracheal instillations, each of 3 mg of hematite alone and no BP. Group 3 consisted of 100 δ and 100 ♀; these remained untreated. This group was otherwise handled in the same manner as Groups 1 and 2.

RESULTS

Survival rates and average weights of the animals in experimental and control groups are given in Chart 1. The animals in Group 1 with a high tumor incidence had a considerably shortened lifespan; among the females of Group 2 (hematite controls), a few cases of intercurrent diseases lowered somewhat the total survival. The average weight was only significantly lowered in the males of Group 1, affected by the early development of respiratory tract tumors.

Preliminary studies have provided a general knowledge of the penetration, distribution and effects of the particulate carcinogens administered using the present method. Serial sacrifices at different times after the intratracheal instillations of dust suspensions showed that hematite particles penetrate freely through the walls of the respiratory bronchioles and alveoli and are then phagocytized by macrophages which form clusters of various sizes. A moderate histiocytic reaction follows around the dust deposits, and then subsides within a few days, only to leave small macrophagic nodules with no evidence of necrosis or other tissue reaction for the remaining life span of the hamsters (Figs. 3–6). This reaction is essentially similar to that previously observed in rats in similar conditions of treatment with hematite (37, 48). When BP is added to the hematite, the penetration, distribution, and tissue reactions are the same. Histoprocated nodules and may be responsible for all of the tumors observed in the respiratory tract in the present experiments.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment*</th>
<th>Sex</th>
<th>Initial number of hamsters</th>
<th>No. of hamsters autopsied</th>
<th>Tumors of the respiratory tract</th>
<th>Other tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Benzo[α]pyrene + Hematite, 15 times</td>
<td>δ</td>
<td>30</td>
<td>28</td>
<td>14</td>
<td>1b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>♀</td>
<td>30</td>
<td>27</td>
<td>21</td>
<td>3b</td>
</tr>
<tr>
<td>2</td>
<td>Hematite alone, 15 times</td>
<td>δ</td>
<td>20</td>
<td>20</td>
<td>0</td>
<td>2d</td>
</tr>
<tr>
<td></td>
<td></td>
<td>♀</td>
<td>24</td>
<td>21</td>
<td>0</td>
<td>2b</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>δ</td>
<td>100</td>
<td>90</td>
<td>0</td>
<td>20f</td>
</tr>
<tr>
<td></td>
<td></td>
<td>♀</td>
<td>100</td>
<td>86</td>
<td>0</td>
<td>13g</td>
</tr>
</tbody>
</table>

Tumor induction in Syrian golden hamsters following intratracheal administration of dust suspensions.

* Intratracheal instillations were given once weekly. Group 1: each dose consisted of 3 mg benzo[α]pyrene + 3 mg hematite in 0.2 ml saline. Group 2: each dose consisted of 3 mg hematite in 0.2 ml saline.

1 forestomach papilloma.

3 forestomach papillomas.

1 forestomach papilloma and 1 retroperitoneal fibroma.

1 malignant lymphoma (histiocytic) and 1 cholangiocarcinoma.

7 malignant lymphomas, histiocytic, 6 forestomach papillomas, 2 liver hemangiomas, 1 thyroid adenoma, 1 thyroid carcinoma, 1 dural melanocytoma, 1 adrenal cortical adenoma and 1 adrenal cortical carcinoma.

2 malignant lymphomas, histiocytic, 2 forestomach papillomas, 2 cholangiomas, 2 cholangiocarcinomas, 1 kidney adenocarcinoma, 1 malignant Schwannoma of facial nerve, 1 adrenal cortical carcinoma, 1 bilateral adrenal cortical adenoma, 1 uterus leiomyosarcoma, and 1 uterus adenocysticoma.

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Table 2

<table>
<thead>
<tr>
<th>Tumor incidences</th>
<th>Males</th>
<th>Females</th>
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<tbody>
<tr>
<td>Initial number of animals</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Effective number of animals</td>
<td>19</td>
<td>21</td>
</tr>
<tr>
<td>Survivors at the end of the exposure (16 weeks)</td>
<td>11</td>
<td>21</td>
</tr>
<tr>
<td>Total number</td>
<td>14</td>
<td>21</td>
</tr>
<tr>
<td>Tumor-bearing animals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent of effective No. of animals</td>
<td>73%</td>
<td>100%</td>
</tr>
<tr>
<td>Percent of survivors after 16 weeks</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>Total number of tumors</td>
<td>28</td>
<td>38</td>
</tr>
</tbody>
</table>

Sites and types of lesions

<table>
<thead>
<tr>
<th>Site</th>
<th>Tumor type</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larynx</td>
<td>Carcinoma, squamous cell</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Squamous metaplasia alone</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Papilloma, squamous cell</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Polyp</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Trachea</td>
<td>Carcinoma, squamous cell</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Carcinosarcoma</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Fibrosarcoma</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Bronchi</td>
<td>Squamous metaplasia alone</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Squamous cell</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>Carcinoma, Anaplastic</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Adenocarcinoma</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Adenoma</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Adenomatoid lesion</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Alveoli</td>
<td>Alveolar squamous metaplasia</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

Tumors of the respiratory tract in hamsters given benzo[a]pyrene intratracheally for 15 weeks (Group 1).

Animals that died in the first 6 weeks of treatment or that were lost because of postmortem changes or cannibalism were discarded.

Logically no acute toxic or necrotizing effects of BP were noted even with high doses. Using UV fluorescence microscopy, the particulate carcinogen, attached to the carrier dust, is seen to penetrate and to be phagocytized by macrophages. Within a few hours from the administration, BP appears to dissolve in the cells and to spread out to adjacent tissues reaching the mucosa of the large bronchi which appears brightly fluorescent.

The main findings are summarized in Table 1.

Group 1.—The hamsters treated with 15 weekly intratracheal instillations, each of 3 mg BP and 3 mg hematite, showed the dust well distributed in all lobes and collected in the typical clusters of macrophages around bronchiolar walls. Other chronic inflammatory changes were only occasionally seen, particularly in some animals in which tumors of the trachea or bronchi obstructed the lumen producing atelectasis, acute bronchitis, and occasionally interstitial pneumonitis in the areas distal to the obstruction.

A high incidence of tumors of the respiratory tract was induced, as shown in Table 2. The total number of tumor-bearing hamsters and of induced tumors is higher in females than in males, but when the cumulative total number of tumors observed is plotted against time (Chart 2), the distribution patterns for males and females appear quite similar. Males actually started showing their tumors earlier than females, but then all the males were dead by the 45th week while the last females lived up to 60 weeks; a few males died without tumors in the first weeks of the experiment, before the end of the treatment. The last of the 15 instillations was given at the end of the 14th week of experiment, before the end of the treatment. Therefore, the exposure of the lungs to BP lasted about 16 weeks. All the animals that died after the 16th week had one or more tumors of the respiratory tract; therefore, the incidence of these tumors after the end of the exposure was 100% in both sexes. The morphology of the tumors is described below.

Control.—No tumors of the respiratory tract were found in
any of the control groups. In Group 2, treated with hematite alone, 2 males showed peripheral bronchiolar adenomatoid lesions similar to those described below for Group 1, but very small. The incidence of other tumors was within the limits of variation normally seen in our colony of hamsters.

**Morphologic Findings.** The general distribution of dust particles in the lungs following intratracheal administration has been outlined above. At the end of 15 weekly instillations (Fig. 5), the dust appears collected by clusters of macrophages mostly around the wall of respiratory bronchioles. The topical reaction at the site of deposition of the dust does not develop any further (Fig. 6). This basic picture is the same for the groups receiving hematite alone, as well as for those receiving hematite with BP.

The lesions attributable to the action of the carcinogen (Group 1) range from hyperplastic changes of the tracheobronchial mucosa to extensive tumors. Their incidence is reported in Table 2; their morphology will be presently described.

Almost all the lesions appear to originate in the mucosa of the respiratory tree, from the larynx and the trachea down to the bronchial and bronchiolar epithelial lining.

**Hyperplasia** of the bronchial or tracheal epithelium was observed in several cases with various degrees of intensity. In some instances it showed rather atypical features (Fig. 7).

**Squamous metaplasia** developed as one of the earliest induced changes: it was found as early as at the 7th week of treatment in the trachea and at the 9th week in the bronchi; only occasional instances were found after the 21st week. It occurred in patches in the tracheal or bronchial epithelium and, in most cases, it showed considerable keratinization (Figs. 8–10). Although most instances were seen in the large bronchi and the trachea, patches of squamous metaplasia occurred in a few cases in the bronchiolar and alveolar epithelium. When metaplasia was found to be part of a neoplastic lesion, it was not counted separately.

**Bronchogenic carcinoma** was the most frequent type of tumor induced. Several hamsters showed more than one bronchogenic carcinoma; thus, 8 males bore a total of 13 such tumors and 11 females a total of 19. They were found as early as at the 13th week of treatment and continued to be found until the last animals were autopsied. They ranged in size from microscopic lesions to large masses involving most of a lobe. They were found most frequently in the proximal parts of the bronchial tree: the stem bronchi and the first divisions of the bronchial tree: the stem bronchi and the first divisions of the bronchial tree. The lesions attributable to the action of the carcinogen were found particularly in carcinomas growing into the tracheobronchial lumen. In many instances the tumors clearly were derived from the bronchial epithelium (Figs. 23, 29). Certain anaplastic carcinomas showed areas with definite differentiation either of the squamous cell type or, in fewer instances, of the adenoscarcinomatous type, or of both types together (Figs. 25–28).

Anaplastic carcinomas appeared at a later time in the experiment than the squamous variety: the first ones were found at the 21st week in males and at the 38th week in females. As in human pathology the distinction between the less differentiated squamous carcinomas and the anaplastic carcinomas is somewhat arbitrary. The observed anaplastic carcinomas were all of the large cell type (Figs. 24, 26); no tumor has been found resembling the oat-cell carcinoma of human pathology. The cellular patterns observed showed considerable variety, even in the same tumor (Figs. 22–24, 25–28); spindle-cell patterns were found particularly in carcinomas growing into the tracheobronchial lumen. In many instances the tumors clearly were derived from the bronchial epithelium (Figs. 23, 29). Certain anaplastic carcinomas showed areas with definite differentiation either of the squamous cell type or, in fewer instances, of the adenoscarcinomatous type, or of both types together (Figs. 25–28).

Adenocarcinomas were the last type to be found and the least frequent. They showed formation of acini and tubules with different degrees of differentiation, some with active mucus secretion (Figs. 20, 21, 30, 31).

**Bronchial adenomas** were found in a few cases; a small adenoma was found in each of 3 males and 2 adenomas in one female. Only one of those found in the female was large and similar to those observed in mice (Figs. 32, 33). The others were smaller, with poorly defined edges (Fig. 34). They all appeared to derive from outgrowths of the epithelium of small bronchioles.

**Bronchiolar adenomatoid lesions** were seen as early as the 7th week of treatment and occurred throughout the experiment. They appeared as proliferations of the bronchioles to form an adenomatoid pattern lined by columnar or cuboidal cells (Fig. 35). Their size varied considerably; some showed only a few acinar formations around a bronchiole and others spread extensively around the bronchiole involving the adjacent alveoli. They were occasionally found in controls given dust alone. None were found in the untreated controls.

**Tumors of the trachea and larynx.** Papillomas and carcinomas of the trachea were the earliest tumors found in the experiment (9th week in males and 14th week in females). They appeared, in most instances, as papillomatous growths partly or almost totally obstructing the tracheal lumen (Fig. 36). A few carcinomas were of the infiltrative type (Fig. 37). Except for a tracheal polyp in a male, all these tumors were of the squamous cell type; in a couple of instances tubular features were found in some areas of a carcinoma. The 2 laryngeal tumors were also squamous cell carcinomas, poorly differentiated. One carcinosarcoma, with both carcinomatous and sarcomatous tissues intricately mixed, and two fibrosarcomas of the trachea were found. The significance of the tracheal tumors will be discussed below.

No metastases of the respiratory tract tumors were observed in the present material. Metastases from bronchogenic carcinomas were observed in a few cases in subsequent experiments (44) where they were found in the lungs (as small scattered nodules sometimes showing arterioles plugged by tumor cell emboli) and in the regional lymph nodes.
The sequence of changes in the bronchial epithelium, suggested by the observation of the present material, appears to begin with very early hyperplastic changes, sometimes markedly atypical. They were also described following instillations of colloidal suspensions of 7,12-dimethylbenz[a]anthracene (4, 49). Squamous metaplasia also appears quite early and develops in patches; again it can show more or less atypical features. From squamous areas the earliest neoplastic plugs appear to invade the underlying connective tissue. Most bronchial carcinomas in our series clearly show areas in which there is continuation from normal epithelium to hyperplastic and/or metaplastic areas and to fully neoplastic invasive tissue. Squamous cell and anaplastic carcinomas were the most frequent types, as in man, but some adenocarcinomas were also induced. It has been suggested (21) that adenocarcinomas in man may be unrelated to exposure to environmental respiratory carcinogens; it is of interest to note that they were induced by direct exposure to BP in our animals, but that their frequency remained considerably lower than that of squamous or anaplastic tumors.

The tracheal tumors were relatively frequent and appeared early. One might suspect that in their induction a role might be played by repeated mechanical injury during the intratracheal instillations. But we have come to consider this explanation unlikely for the following reasons: (a) the cannulas used for the instillations had blunt ends and we found absolutely no microscopic evidence of any scratching or wounding of the tracheal mucosa; (b) there is no deposition of dust particles in the tracheal walls; and (c) the carcinogen is eluted from the areas of dust deposition and flows toward the hylum, reaching the large bronchi where most tumors arise, and then the tracheal wall. In this respect the tracheal tumors in these experimental conditions could be considered as having the same pathogenetic significance as the bronchial tumors.

A more detailed morphologic classification and an evaluation of the relative frequency of the various types of tumors and of their distribution will be attempted when the cumulative results of several other experiments will provide us with a much larger total number of cases.

The peribronchial adenomatoid lesions are still somewhat difficult to interpret. They have been seen in control animals and have been described in different pathologic conditions of the lungs, particularly in the course of chronic inflammation and fibrosis and following exposure to influenza viruses in mice (20). However, their incidence appears greatly increased and their appearance accelerated by the carcinogen. They have been clearly described by Herrold and Dunham (15) in their experiments on hamsters with BP in Tween solution, and also reported by Shabad in rats (49). In the material of the experiments presented here, we did not find sufficient evidence to claim that any of the tumors originated from such lesions; however, in further studies, some instances of direct extension of peripheral proliferative lesions into neoplastic areas were observed (44).

The pathogenetic mechanisms involved in the induction of lung cancer in the experimental model described here present a number of problems for discussion. Some of these problems appear similar to those posed by human lung cancer. The carcinogens entering into the respiratory tract reach its peripheral...
portion and penetrate into the tissues, together with their carrier particles, through the flat epithelial lining of the respiratory bronchioles and alveoli. The particles are phagocytized by clusters of macrophages near the sites of penetration, and the macrophages retain them for some time as shown by UV fluorescence microscopy. The carcinogen is then eluted out by plasma with a mechanism comparable to that described by Falk et al. (9, 10) and by Kotin (17). Halos of BP fluorescence can in fact be seen around the macrophages. However, most tumors in our experimental model, as well as in human pathology, arise from the mucosa of the proximal parts of the tracheobronchial tree, including the large bronchi, the stem bronchi, and the trachea, rather than from the peripheral (bronchiolar-alveolar) parenchyma. The cells that are first hit by large amounts of the carcinogen are connective tissue cells, macrophages, fibroblasts of the bronchiolar wall, alveolar septa, and capillary endothelia: yet no pulmonary sarcomas were ever seen in our experimental animals and these tumors are extremely rare in man. The carcinogen, eluted out by plasma, then appears to spread out. Kotin et al. (19) showed that radioactivity from BP-14C, instilled intratracheally in rats, is recovered in various other organs and it appears in feces and urine within a few hours. Preliminary observations by UV fluorescence microscopy have allowed us to follow the elution of BP from the dust stored in the peribronchiolar macrophages and to follow its flow through the interstitial tissue to reach the mucosa of the larger bronchi and trachea. The elution of the carcinogen and its retention in the lungs are influenced by the original vehicle (19) and other factors, such as dose level and particle size, presently under study in our laboratory (39, 42, 46). A reason for the prevalent proximal location of the tumors could be that the proximal segments of the respiratory tract, where the eluted carcinogen converges from all the peripheral lobules, represent areas of maximal tissue exposure to the carcinogen. Both time and concentration must be considered in assessing the effective cellular exposure at different levels of the respiratory tract. Another possibly determinative factor could be the susceptibility of the different cells of the respiratory tract as represented, for example, by their different binding capacity for the carcinogen. Studies of the distribution of BP in cells of the different portions of the respiratory tract are under way in our laboratory. The carcinogen is eluted out of the carrier dust at sites somewhat remote from those of origin of the tumors; in our model, increasing the amount of inert dust does not influence the recovery rate of BP (46). The dust, therefore, plays a very important role in the essential initial phase of penetration of the carcinogen into the pulmonary tissue but does not appear to play any direct topical role in the neoplastic transformation of the epithelial cells of the tracheobronchial mucosa.

The conditions of exposure to respiratory carcinogens for lung cancer induction encompass a variety of physical, chemical, and physiopathologic factors (8, 18, 42). The understanding of their role is the main object of our studies with the present method.

To date, the conclusions acquired from our studies can be summarized as follows. (a) The present method has been found adequate to induce a high incidence of tracheobronchial tumors (up to 100% of the survivors after treatment). (b) Most of the induced tumors clearly show their origin from the tracheobronchial epithelium and include a large proportion of bronchogenic carcinomas of the 3 main types seen in human pathology (squamous cell, anaplastic, and adenocarcinoma). (c) No extensive necrosis and no chronic inflammation other than dust phagocytosis are involved in the lung response to the administration of the carcinogen. Therefore, these factors are not required as an essential part of the pathogenetic mechanism of bronchogenic cancer. (d) No special solvents or colloids are required for the administration of the carcinogen carried by dust particles; the method of administration in particulate form bears close resemblance to the conditions of human exposure to respiratory carcinogens.

Adequate penetration of the carcinogen into the lung tissues and its spread from there to reach the bronchial epithelium by diffusion seem to be the key factors in the induction mechanism of bronchogenic tumors. A number of studies on the physical factors that control the penetration, phagocytosis, elution, and diffusion of the carcinogen are presently under way in our laboratory. The methodology reported here gives us an opportunity to investigate the role in lung carcinogenesis of such factors as: the particle size of the carcinogen and of the carrier dust; the surface characteristics, adsorptivity and solubility of the particles; the role (if any) of the load of inert dust in the lung; the doses of carcinogen and the periods of exposure; the modes of penetration, distribution, and spread of carcinogens in the lung; the role (if any) of chronic inflammation that can now be superimposed to a model that does not imply it; the role of additional factors such as other chemicals or viruses; and the role of certain conditions of the host and of factors affecting them in relation to the susceptibility to carcinogenesis of the bronchial epithelium as, for example, the role of cellular differentiation in the bronchial epithelium as modified by vitamin A administration (45).

It is hoped that the present experimental model will contribute a useful tool in the understanding of the histopathogenesis of cancer of the lung and in the search for its causative mechanisms.

ACKNOWLEDGMENTS

We wish to thank Dr. Philippe Shubik for his constant interest and encouragement; Dr. William Lijinsky for advising on chemical problems; Dr. Béla Toth for observing the untreated control group jointly with us (54); Mr. Seth A. Borg, Dr. Marvin I. Grote, and Dr. David B. Karp for their collaboration during their research fellowships as medical students in this laboratory; Mrs. Elena Sileikis for her technical assistance in the experimental work; Mr. Manuel Sueiro and Mrs. Helen Cummins for the histologic preparations; and Mr. Andrew Washington and Mr. Gene Downs for their help in the photographic work.

We also wish to thank the Fisher Scientific Co. for supplying us with the particle size analysis of the hematite dust.

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Fig. 1-37. All sections were stained with H & E, except for Fig. 31. Sections in Figs. 5-37 are all from hamsters of Group 1, treated with 15 intratracheal instillations of benzo[a]pyrene-hematite dust; the time of death is given in weeks after the beginning of the experiment.

Fig. 1. Dust sample made of benzo[a]pyrene (BP) and hematite particles (1:1 by weight). 1a, transmitted tungsten light; 1b, polarized light showing birefringence of both BP and hematite; 1c, UV light showing fluorescence of BP particles. (For the smaller particles, the fluorescence is faint and hardly visible in the photograph.) X 500.

Fig. 2. Procedure for intratracheal instillation (see text). The dust suspension is kept on a magnetic stirrer (lower left); the hamster's throat is illuminated by a headlamp (upper right).

Fig. 3. At 24 hr after single instillation, most dust has penetrated into the walls of respiratory bronchioles and alveoli; no penetration is observed through the terminal bronchioles. X 56.

Fig. 4. At 48 hr after single instillation, the dust appears phagocytized. A moderate histiocytic reaction has developed around dust deposits. X 97.

Fig. 5. Dust distribution at the end of the 14th week of treatment, after a course of 15 instillations. The dust appears still located in the walls of respiratory bronchioles and alveoli. No inflammatory reaction other than the accumulation of macrophages at the sites of dust deposition. X 30.

Fig. 6. Dust distribution at the 45th week, i.e., 31 weeks after the last instillation. The dust is still mostly deposited in its original sites and no further inflammatory reaction has developed. Note dust particles aligned in the peribronchiolar lymphatics, and some in the lumen, representing the two main clearance mechanisms for inert dusts. X 55.

Fig. 7. Bronchial hyperplasia with highly atypical changes, including altered polarity and pleomorphic nuclei. X 375.

Fig. 8. Bronchial squamous metaplasia (14 weeks). X 190.

Fig. 9. Tracheal squamous metaplasia with hyperkeratinization. (9 weeks). X 280.

Fig. 10. Bronchial squamous metaplasia with hyperkeratinization and atypical changes; some dust has remained trapped in the bronchial lumen between keratin layers. Some nests of atypical cells below the basement membrane (arrow) indicate a very early malignant invasive change. Same animal as in Fig. 11, (δ, 13 weeks). X 110.

Fig. 11. Early invasive bronchogenic squamous cell carcinoma (δ, 13 weeks; same animal as in Fig. 10 in another part of the bronchial tree). X 105.

Fig. 12. Squamous cell carcinoma arising from the epithelium of the main bronchus of the right lobe and infiltrating through the basal membrane (δ, 57 weeks). X 135.

Fig. 13. Bronchogenic squamous cell carcinoma arising from secondary bronchus in the left lobe (δ, 17 weeks). X 23.

Fig. 14. Detail of Fig. 13, showing the invasive patterns of neoplastic squamous cells and their origin from the bronchial mucosa. X 90.

Fig. 15. Bronchogenic squamous cell carcinoma arising from and occluding the right stem bronchus, and showing an unusually high degree of keratinization (δ, 29 weeks). X 13.

Fig. 16. Detail (a) from Fig. 17, showing bronchogenic squamous cell carcinoma arising from atypical metaplastic keratinizing squamous bronchial epithelium (δ, 29 weeks). X 270.

Figs. 17-21. Fig. 17 (δ, 29 weeks, X 13) shows a large squamous cell carcinoma invading most of the right lower lobe and protruding into the right stem bronchus. The tumor exhibits a variety of structural patterns, such as areas with cord-like patterns (b: Fig. 18, X 85) and large areas with keratinized horny pearls (c: Fig. 19, X 85). In the lower portion of the same lobe there is a small adenocarcinoma adjacent to the squamous cell carcinoma (d: Figs. 20, X 110 and 21, X 140). The remaining parenchyma appears alveolated and congested.

Figs. 22-24. Panoramic view of the right lower lobe (Fig. 22, δ, 49 weeks, X 17), with discrete dust deposits and a small anaplastic carcinoma of the main bronchus showing invasion from the bronchial epithelium (a: Fig. 23, X 145) and the large-cell pattern of the tumor with some multinucleated giant cells (b: Fig. 24 X 225).

Fig. 25. Composite panoramic picture showing the trachea, right stem bronchus, upper, middle, and lower right lobes (two sections of the lower right lobe are mounted side by side). (δ, 43 weeks, X 11). A large, mostly anaplastic carcinoma infiltrates the wall of the right main bronchus (a) and expands into the lower lobe, (b) where it forms a large nodule (c); the infiltration extends also to the middle lobe (d). Serial sections show continuity of the tumor from its origin in the right stem bronchus to the areas in the lobes showing anaplastic, squamous and adenocarcinomatous patterns (see Figs. 26-28).

Fig. 26. Detail of Fig. 25 from right middle lobe (area d), showing anaplastic features. X 225.

Fig. 27. Detail of Fig. 25 from right lower lobe (area c), showing tubular adenocarcinomatous pattern (X 200).

Fig. 28. Detail of Fig. 25, from right lower lobe near the bronchial origin of the tumor (area b), showing squamous differentiation. X 200.

Fig. 29. Anaplastic carcinoma arising from the main bronchus of the left lobe (δ, 57 weeks, X 105).

Fig. 30. Adenocarcinoma arising from the right stem bronchus near the tracheal bifurcation (left upper corner) and infiltrating the carina with dilated tubular pattern. (δ, 38 weeks, X 53).

Fig. 31. Adenocarcinoma invading the whole left lobe and showing tubular formations lined by mucus-producing neoplastic epithelium (δ, 38 weeks, periodic acid-Schiff, X 107).

Figs. 32, 33. Adenoma in the right upper lobe (δ, 49 weeks) with a pattern similar to that of lung adenomas in mice. Note the dust particles trapped between the tubular structures. (Fig. 32, X 16; Fig. 33, X 234).

Fig. 34. Small adenoma around a bronchiole in the right upper lobe (δ, 45 weeks). X 58.

Fig. 35. Bronchiolar adenomatoid lesion, showing proliferation of columnar cell epithelium in respiratory bronchioles and adjacent alveoli. (δ, 13 weeks). X 94.

Fig. 36. Squamous cell papilloma from the upper third of the trachea showing marked keratinization; (δ, 38 weeks). X 30.

Fig. 37. Squamous cell carcinoma in the lower third of the trachea infiltrating the tracheal wall. (δ, 21 weeks). X 145.
Induction of Bronchogenic Carcinoma

Images 7, 8, 9, 10, 11, 12
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A Method for the Experimental Induction of Bronchogenic Carcinoma

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