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TESTS FOR EFFECT OF ASBESTOS ON BENZO[a]PYRENE CARCINOGENESIS IN THE RESPIRATORY TRACT*

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In a preceding paper,[1] it was noted that exposure to asbestos has been associated with an increased incidence of pulmonary carcinomas and mesotheliomas in man, but that experiments by many investigators, including ourselves, have failed to demonstrate carcinogenic activity of asbestos in animals, except after intrapleural injections of large doses. The failure to find malignant tumors in the lungs of animals after intratracheal or inhalation exposure to various preparations of asbestos suggested an hypothesis that pulmonary cancers associated with asbestos in man might not be attributable to any primary carcinogenic action of asbestos fibers, but rather to a promoting or cocarcinogenic effect of asbestos upon some carcinogen(s) present in human lungs and lacking in lungs of animals. It seemed possible that carcinogens present in cigarette smoke might be involved. Experiments now reported were designed to explore this hypothesis.

Benzo[a]pyrene was selected as a carcinogen known to be present in cigarette smoke.[2] Selection of this carcinogen was prompted because Herold and Dunham[3] had reported induction of tracheobronchial papillomas and carcinomas in hamsters after repeated intratracheal injections of benzo[a]pyrene suspended in aqueous Tween 60.

Two varieties of asbestos were employed. One was a preparation of chrysotile provided by the late E. J. King of the London Postgraduate Medical School. This material was reported to have an average fiber length of 18 microns. No cancers had resulted in the lungs of rats given intratracheal injections of this material by King and his associates.[4] The other preparation was a sample of amosite prepared by a method developed in our laboratory[5] and found to have an average fiber length of 17.6 microns. In our laboratory, mesotheliomas have been found in hamsters after intrapleural injection of this preparation of amosite ("Sample 7"), but no tumors have yet been found after repeated intratracheal injections of this preparation into hamsters.[1]

METHODS

Male golden Syrian hamsters were obtained from Lakeview Hamster Colony, Newfield, N. J., as weanlings. The animals were one to two months

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old when tests were started. They were housed in groups of five or ten in wire mesh cages. They were fed a commercial pelleted feed ("GLF Diet for Hamsters," W. F. Fisher, Bound Brook, N. J.) supplemented three times a week with apples, carrots or spinach. Water was available *ad libitum*.

Benzo[a]pyrene, obtained from Eastman Organic Chemical Company, Rochester, N. Y., was prepared in one or 0.5 per cent suspension in one per cent aqueous polyoxyethylene (20) sorbitan monostearate ("Tween 60"). Chrysotile or amosite preparations, mentioned above, were added to the suspension as indicated for certain injections. The suspensions were freshly prepared on each day that injections were made.

Intratracheal injections were made according to the method described by Herrold and Dunham[3] for hamsters, using sodium pentobarbital anesthesia ("Diabutal"). A 1+10 dilution of veterinary Diabutal solution was used in amounts containing 0.65 mg. sodium pentobarbital per gram of body weight.

Insertion of the pipette into the trachea rather than into the esophagus was checked before each injection by compressing the chest and observing the rise of liquid in the pipette resulting from compression of the lungs. The injections were made on a weekly schedule continuing until the desired amounts of asbestos and benzo[a]pyrene had been administered.

When animals died or were sacrificed, the larynx, trachea and lungs were removed and examined under a dissecting microscope at 7× magnification. A cut was made down the dorsal aspect of the larynx and trachea and was extended into the bronchi as far as they could be followed into the parenchyma of the lungs. The exposed mucosa of the larynx, trachea and bronchi was examined under the dissecting microscope. Descriptions of the gross specimens were recorded. The larynx and trachea, right and left lung were then fixed in 10 per cent formalin containing 2 per cent sodium acetate. Sections were cut and stained with hematoxylin and eosin. Lungs were sectioned in their long axis through the hilum.

Findings

Two experiments were carried out. All lesions recognized as tumors in gross specimens by examination at 7× with a dissecting microscope proved to be papillomas or carcinomas when studied in histological sections. Some small neoplasms, not detected in the gross, have been found in histological sections. Histological studies of all specimens have not yet been completed. Tabulation of tumors in the present report is therefore restricted to those that were detected by examination of gross specimens and then confirmed in histological sections.

Experiment 1

This experiment was a pilot test designed primarily to learn the time

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of appearance of tumors in the respiratory tract of hamsters after repeated intratracheal injections of 0.5 mg. benzo[a]pyrene at one per cent suspension in Tween 60 with and without chrysotile. The amount of chrysotile per injection was 0.25 mg. Information on time of appearance of tumors was desired in order to estimate the time for sacrifice and examination of comparative groups in subsequent work.

Fifty hamsters were divided into five treatment groups of ten animals each. They were given intratracheal injections once a week for 25 weeks. Some individuals died, in most instances from asphyxiation immediately after injections, before receiving the full course. The volume of each injection was 0.05 ml. Treatment and results were as follows:

Group 1. Benzo[a]pyrene. No tumors were found in five hamsters that died at 20, 86, 88, 105 and 138 days, respectively.

On the 174th day, a hamster was observed to have labored respiration. The animal held his mouth open, was breathing in slow, deep gasps, and seemed exhausted by the labor of breathing. He had lost 30 grams in weight in the preceding week. He was sacrificed. The larynx was found to be nearly occluded by a 3 × 3 × 2 mm. mass of translucent gray tissue seated by a broad base on the anterior wall. Microscopically, this proved to be an epidermoid carcinoma.

In a hamster found dead on the 184th day, a 5 × 5 × 0.5 mm. rugose mass was found on the wall of the trachea. The left bronchus was almost ob-

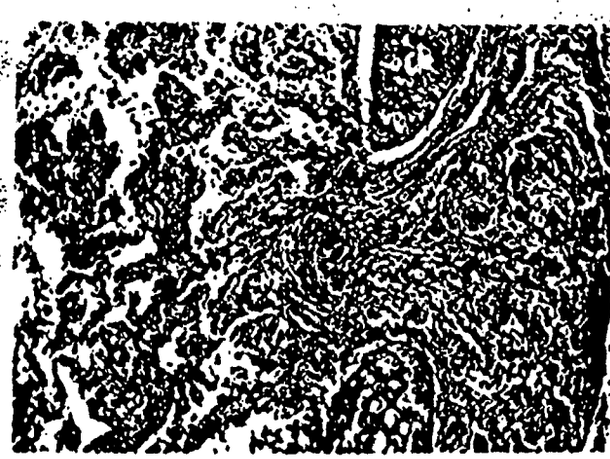

FIGURE 1. Papillary adenocarcinoma bulging into lumen of trachea (*left*) and invading between two tracheal cartilages (*center*) to infiltrate wall of trachea (*right*). In the wall, the tumor has undergone epidermoid change. 184 days after start of intratracheal injections of benzo[a]pyrene. 100 ×.

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FIGURE 2. Epidermoid carcinoma bulging into lumen of trachea (*left*) and invading between two tracheal cartilages (*center*) to infiltrate wall of trachea (*right*). In the wall, the tumor is seen as a scirrhous growth. 176 days after start of intratracheal injections of benzo[a]pyrene and chrysotile. 100 ×.

structed by a 3 × 2 × 2 mm. mass. Microscopically, the tumor in the trachea proved to be a papillary adenocarcinoma (FIGURE 1), and the tumor in the bronchus was an epidermoid carcinoma.

Two hamsters were sacrificed at 190 days, and the last individual in this group at 214 days. All seemed in excellent condition at the time of sacrifice. No tumors were found in the two killed at 190 days, but in the 214-day animal a 2.5 × 2 × 2 mm. epidermoid carcinoma obstructed the right bronchus.

Group 2. Benzo[a]pyrene plus chrysotile. Three animals died without tumors at 1, 98, and 108 days respectively. On the 173rd day, a hamster was found dead with pneumonia of the right lung and an epidermoid carcinoma in the right bronchus.

On the 176th day, a hamster was observed to show rapid, labored respiration. He had lost 22 grams in weight in the preceding week. He was sacrificed. The trachea was constricted by a 5 × 4 × 3 mm. mass which had encircled it and invaded its wall as shown by a cut surface which disclosed four tracheal cartilages buried in the tumor. Microscopically, this was a scirrhous epidermoid carcinoma that formed lamellated keratin. In the same animal, a 1.5 × 1 × 0.5 mm. broad-based translucent gray mound was seen under the dissecting microscope to be seated on the wall of the right bronchus. This proved to be a less differentiated epidermoid carcinoma.

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Also on the 176th day, another hamster who showed labored respiration and loss of 38 grams in weight was sacrificed. He had a 3 × 2.5 × 2 mm. firm white mass in the wall of the trachea, which proved to be a scirrhous epidermoid carcinoma (FIGURE 2). In the mucosa of the left bronchus, there was a discoid opaque white thickening 1 mm. in diameter and 0.5 mm. thick in which three yellow spots were visible under the dissecting microscope. Keratinized pearls found in histological sections of this lesion accounted for the yellow spots. Serial sections of this lesion revealed no invasive growth, but irregularity in size and shape of cells and marked hyperchromatism led us to classify it as carcinoma *in situ*.

On the 190th day, another hamster with labored respiration and two [illegible] who were not remarkable and seemed in excellent condition were sacrificed.



FIGURE 3. Papilloma seated by a narrow base on wall of trachea and bulging into lumen. 190 days after start of intratracheal injections of benzo[a]pyrene and chrysotile. 100 ×.

The last member of the group was killed on the 214th day because he was *gasping* for air. He had slowly lost 35 grams in weight in the preceding three months. Infiltrating carcinomas were found in the respiratory tracts of all four of these animals. The first had a 5 × 5 × 4 mm. mass in the trachea that had invaded the wall and invested the esophagus. The second had carcinomas in the larynx (1.5 × 1.5 × 0.5 mm.), trachea (2.5 × 2 × 1 mm.) and right bronchus (3 × 2.5 × 2 mm.). The third had three discrete lesions in the trachea and one in the right bronchus, each about 1 mm. in diameter. One of the lesions in the trachea of this animal was a papilloma

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(FIGURE 3). The 214-day hamster had a 1 mm. papilloma in the trachea and a 5 × 3 × 5 mm. mulberry-like mass in the right bronchus, which was a keratinizing epidermoid carcinoma.

Group 3. Chrysotile. No tumors were found in any animals of this group. Six lived longer than the time of detection (173 days) of the first tumor in the preceding groups. These six were sacrificed for examination in pairs at 177, 220 and 249 days, respectively.

Group 4. Solvent control (aqueous Tween 60). No tumors were found in any animals of this group. Two were sacrificed for examination at 220 days and the remaining eight at 300 days.

Group 5. Untreated controls. No tumors were found in any animals of this group. All were sacrificed for examination between 220 and 300 days.

TABLE 1

TRACHEOBRONCHIAL TUMORS IN HAMSTERS 173 TO 214 DAYS AFTER START OF WEEKLY INTRATRACHEAL INJECTIONS OF BENZO[A]PYRENE WITH AND WITHOUT CHRYSOTILE

Treatment	No. of animals bearing tumors/ no. of survivors	No. of tumors	No. of tumors per tumor-bearing animal	No. of tumors larger than 3 mm.
BP	3/5	4	1.3	0
BP + CHR	7/7	15	2.1	3
CHR	0/6	0	—	—
Tween	0/10	0	—	—
None	0/10	0	—	—

BP = Benzo[a]pyrene CHR = Chrysotile

This experiment showed that the dosage schedule of benzo[a]pyrene employed could be counted upon to induce carcinomas in the respiratory tract of hamsters within seven months. It also disclosed that presence of such tumors could be recognized by observation of labored respiration in cases where there was obstruction of the airway. These two pieces of information afforded guides for selection of appropriate times to sacrifice animals for examination in further similar experimentation.

The earliest tumor in this first test was found 173 days after the first injection. In hamsters that lived between 173 days and 214 days after treatment with benzo[a]pyrene alone, respiratory tract tumors were found in three out of five, showing that this dose of benzo[a]pyrene was too large for convenient measurement of any cocarcinogenic action of asbestos. There were tumors in each of seven hamsters examined during the same period after treatment with benzo[a]pyrene (BP) plus chrysotile (CHR). Also, as shown in TABLE 1, the number of tumors per tumor-bearing animal,

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and the size of the tumors was greater in the group treated with BP + CHR than in the group treated with BP alone. Histologically, the most malignant tumors were found in the BP + CHR group. All four criteria thus suggested that chrysotile had promoted benzo[a]pyrene carcinogenesis, but the numbers of animals were too small to permit a decision. As noted above, no tumors were found in the respiratory tracts of untreated hamsters or in those treated with chrysotile alone or with the suspending medium (aqueous Tween 60).

Experiment 2

This experiment was carried out with a reduced total dose of benzo[a]pyrene and an increased dose of asbestos. The asbestos used in this test was a sample of amosite, described at the beginning of this paper. In other experiments it did not induce tumors after repeated intratracheal injections into hamsters. The volume of each injection was doubled to 0.1 ml. to carry the increased amount of asbestos. The concentration of benzo[a]pyrene in the suspension was therefore halved to 0.5 per cent. The amount of benzo[a]pyrene per injection remained the same as in the preceding test, but the total number of injections was reduced.

For Experiment 2, eighty hamsters were given weekly intratracheal in-

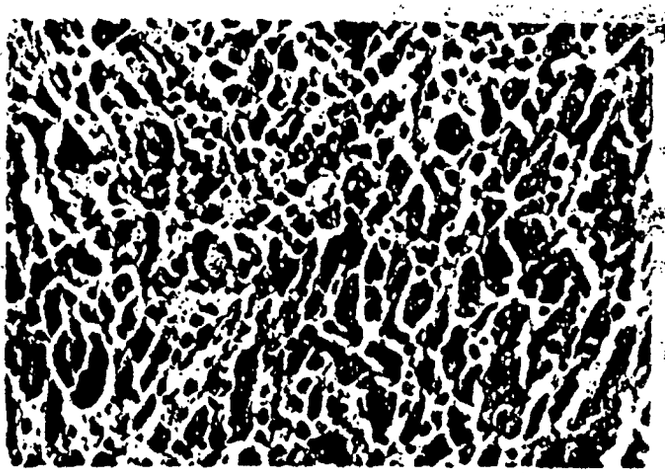

FIGURE 4. Undifferentiated carcinoma found 211 days after start of intratracheal injections of benzo[a]pyrene. This tumor was a 10 mm. spherical mass in apex of left lung. It was a solid, malignant epithelial tumor. The individual cells show considerable variation in size and shape with occasional giant forms and scattered mitotic figures. There is no differentiation in the direction of gland formation or keratinization. 400 ×.

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FIGURE 5. Epidermoid carcinoma filling the lumen of a bronchus near a bifurcation. The uninvolved branch bronchus is in the lower right corner of the photograph. Alveolar spaces are seen at lower center. They are separated from the tumor by a narrow line of normal bronchial mucosa. The tumor occupies the rest of the photograph. 240 days after start of intratracheal injections of benzo[a]pyrene. 100 ×.

jections of 0.5 mg. benzo[a]pyrene in one per cent aqueous Tween 60. In half of these eighty, each injection also contained 2.5 mg. amosite, a tenfold increase in the amount of asbestos over that used in the preceding test. The total number of injections was 16. Hamsters that survived the full course of 16 injections thus received a total dose of 8 mg. of benzo[a]pyrene in contrast to the total dose of 12.5 mg. of benzo[a]pyrene that had been delivered in the 25 injections of the preceding experiment. An outbreak of an endemic disease of hamsters ("wet tail") caused some deaths in both the BP and the BP + amosite groups, but the health of the survivors did not seem impaired as judged by their appearance and weight records. Findings were as follows.

Group 6. Benzo[a]pyrene. No tumors were found in the respiratory tract of a hamster that died 98 days after start of test. In a hamster found dead on the 166th day, a 10 mm. spherical mass lay in the hilum of the right lower lobe. It was an epidermoid carcinoma. In a hamster sacrificed because of labored respiration on the 201st day, a 3 mm. spherical mass with constricted base filled the lumen of the trachea. It was a benign papilloma. Ten more hamsters died between 201 and 217 days. Respiratory

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tract tumors were found in six of them; there were three papillomas and three carcinomas. One of the carcinomas is illustrated in FIGURE 4. On the 240th day, all 27 survivors were sacrificed for examination. Respiratory tract tumors were found in eight of them. Among these eight, there were five papillomas and four carcinomas. One of the carcinomas is illustrated in FIGURES 5 and 6.

Group 7. Benzo[a]pyrene plus amosite. No tumors were found in eight hamsters that died between 30 and 105 days, in nine that died from an outbreak of wet tail in a single cage between 144 and 161 days, or in three that died at 183, 192, and 218 days, respectively. A small carcinoma, lying near the pleura and not detected in the gross, was found in lung sections of a hamster dying on the 223rd day. On the 240th day, all 18 survivors were

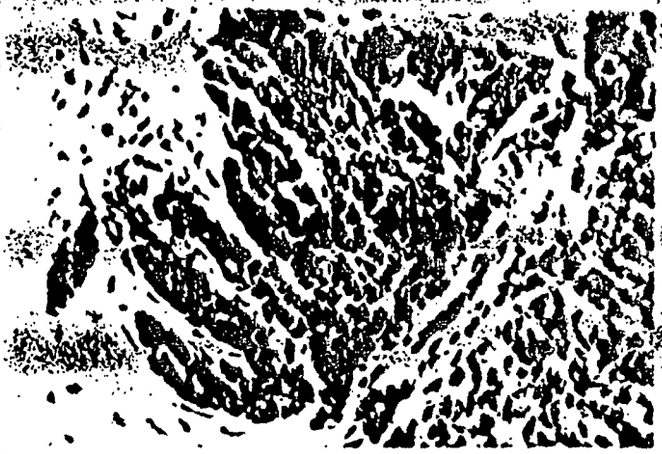

FIGURE 6. Higher magnification of cells of the tumor shown in FIGURE 5. The main mass of the tumor in the bronchus is at the right. The island of cells shown at center is one of many infiltrating downgrowths that penetrated the wall of this bronchus. 400 ×.

sacrificed for examination. Respiratory tract tumors were found in five of them, a single papilloma in each instance.

The finding of tumors in hamsters treated only with benzo[a]pyrene who died prior to 240 days, and the absence of tumors in hamsters treated with benzo[a]pyrene plus amosite who died prior to 240 days, is difficult to compare because the latter group did not live as long as the former. In the substantial numbers of hamsters in the two treatment groups that were all examined at the same time (240 days), the yield of tumors was

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TABLE 2
TRACHEOBRONCHIAL TUMORS IN HAMSTERS SACRIFICED 240 DAYS AFTER START OF 16 WEEKLY INTRATRACHEAL INJECTIONS OF BENZO[A]PYRENE (BP) WITH OR WITHOUT AMOSITE

Treatment	No. hamsters at start of test	No. survivors at 240 days	No. with tumors	Per cent tumors
8 mg. BP	40	27	8	29%
8 mg. BP + 40 mg. Amosite	40	16	5	26%

Mean weights: 145 grams (BP group), 138 grams (BP + Amosite group)

almost identical, 29 versus 26 per cent (TABLE 2). A yield in this range showed that the dosage of benzo[a]pyrene used in *Experiment 2* was well suited to test for any promoting action of amosite. No evidence for any such action was found on the basis of tumors detected by examination of gross specimens under a dissecting microscope and verified in histological sections. Many additional blocks of lungs of animals in this experiment remain to be cut and studied histologically.

Discussion

In *Experiment 1*, the course of 25 weekly injections of benzo[a]pyrene was completed on the 175th day, and tumors were found in 10 of 12 hamsters examined between 173 and 214 days. In *Experiment 2*, the course of 16 weekly injections was completed on the 112th day. It was of interest that tumors were found in this experiment in seven of eleven hamsters examined between 201 and 217 days, but in only eight of 27 examined on the 240th day. This is a difference of approximately 64 versus 30 per cent. The animals examined on the 240th day had received the same dose of benzo[a]pyrene as those examined earlier. They had a longer time to develop tumors, but the proportion that showed tumors was less by half.

This difference suggests several explanations. First, the susceptibility of some individuals may have been greater, and they would then have been the ones that responded promptly with tumors. Second, benzo[a]pyrene may have attained a greater local concentration in some individuals, for example, as a result of the injection being deposited largely in one lobe, and these may have been the ones that developed tumors early. The data, however, are curiously reminiscent of observations by Rous and Kidd[8] on the genesis of tumors in the skin in response to a tar. They found that after repeated painting of this tar onto the ears of rabbits until tumors began to appear, discontinuation of exposure to the tar led to regression

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of many tumors and failure of new tumors to appear. Similar events could explain the results of *Experiment 2*. The data of *Experiment 2* are thus of interest as indicating that exposure of the mucous membranes of the respiratory tract to a limited series of doses of benzo[a]pyrene does not necessarily involve an irreversible progression toward cancer. Since benzo[a]pyrene is a carcinogen known to be present in cigarette smoke, these data are of interest in relation to a decrease in lung cancer risks in smokers who discontinue smoking.

Recently, Harington and Smith[1] reported differences in the ability of different varieties of asbestos to adsorb benzo[a]pyrene. In their studies, chrysotile was most effective and amosite was least effective in adsorbing ability. Adsorption of carcinogens to asbestos fibers may play an important role in any effect of the fibers upon carcinogenesis. Shabad and co-workers[2] have found that admixture of India ink particles to suspensions of benzo[a]pyrene increases retention of this carcinogen in the lungs of rats after intratracheal injection. Their paper summarizes other experiments with rats in their laboratory in which intratracheal injections of another polycyclic hydrocarbon, 9,10-dimethyl-1,2-benzanthracene, failed to induce lung cancers when suspended in physiological saline but did induce lung cancers when suspended in saline containing casein and India ink.

Conclusions

Time of appearance and yields of papillomas and carcinomas in the respiratory tract of hamsters after intratracheal injections of benzo[a]pyrene are reported.

Addition of the chrysotile variety of asbestos to the injections gave results consistent with an hypothesis that this material promoted benzo[a]pyrene carcinogenesis in the respiratory tract. Addition of the amosite variety of asbestos to injections of benzo[a]pyrene did not increase the yield of tumors found in the respiratory tract.

The yield of tumors in the respiratory tract of hamsters treated with a limited series of intratracheal injections of benzo[a]pyrene decreased with time after discontinuation of exposure. This observation is of interest in relation to lung cancer risks in smokers who discontinue smoking.

Further studies of histological material from these experiments are in progress.

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