Quantitative Exposure of Grafted Rat Tracheas to 7,12-Dimethylbenz(a)anthracene

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SUMMARY

A method was developed for continuously exposing tracheal epithelium to measured amounts of carcinogen. Beeswax was the vehicle for sustained release of carcinogen, and tracheas transplanted to s.c. sites were target tissues. In the experiment reported here, transplanted rat tracheas were exposed to a potent carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA).

The rate of release of DMBA from the beeswax carrier within the tracheal lumen approached first order when the initial concentration of carcinogen was high (3200 to 325 μg in a 24.45-mg pellet). With lower concentrations, where the carcinogen was dissolved in the beeswax, initial release was rapid, and most of the carcinogen was delivered within 4 weeks.

At high DMBA dose levels, the entire tracheal epithelium was uniformly replaced by keratinizing squamous metaplasia after 1 week of exposure, and after 2 months, when from 280 to 910 μg DMBA had been delivered, all transplants had developed invasive squamous carcinomas. Sarcomas also developed in 19% of the transplants.

At lower dose levels the epithelial reactions were more varied, and tumor development was more protracted. The lowest DMBA dose presently known to induce carcinomas in this experimental model is 40 μg, which is in the dose range used for tumor initiation in skin carcinogenesis studies in mice.

INTRODUCTION

A major limitation of most respiratory carcinogenesis studies is that the commonly used experimental models do not permit delivery of a known amount of carcinogen to a target of predetermined size in a topographically defined region of the respiratory tract. After a carcinogen is administered i.t. or by inhalation exposure, it is distributed unevenly over large surface areas in various segments of the tracheobronchial tree and the alveolar parenchyma. Subsequently, a redistribution of the material within the respiratory tract occurs, as it is cleared by various mechanisms. Thus not only dose and dose rate but also duration of exposure to the carcinogen are poorly defined. Multiple exposures, which are often required to induce a sufficient tumor response, further complicate this picture. For these reasons several investigators have tried to develop methods for topical application of carcinogens, with varying degrees of success (4, 5, 7).

The development of new localized tumor-induction systems was recently reported from our laboratory (1, 3, 6). One of these, the tracheal transplant model (1-3, 6), is particularly well suited for quantitative studies. Transplanted tracheas serve as the target tissue, and uniform exposure of the entire tracheal epithelium over an extended period of time is effected by incorporating the carcinogen into a beeswax pellet that largely fills the tracheal lumen. The present report describes the first systematic tumor induction study with this new experimental model in which DMBA was the carcinogen. Emphasis is placed on carcinogen release and carcinogen dose-response relationships. A companion paper (8) describes the response of tracheal transplants to benzo(a)pyrene, emphasizing the morphological descriptions and documentation of various preneoplastic and early neoplastic lesions. In principle the epithelial pathological changes induced by these 2 polycyclic hydrocarbons were similar.

MATERIALS AND METHODS

Animals. Ten-week-old inbred Fischer 344 male and female rats, bred and maintained in a barrier facility, were used. They were demonstrated to be free of the common respiratory pathogens of rats, including mycoplasmas.

Tracheal Transplants. The technique for transplanting tracheas (6) was modified to prevent them from curling or contracting and to facilitate insertion of carcinogen pellets. After the donor tracheas were surgically exposed and flushed with Hanks’ balanced salt solution, they were attached at either end to a 3.0-cm length of sterile polyethylene tubing with silk sutures. The tracheas, from the 2nd tracheal ring to the bifurcation, were then excised and held in Hanks’ solution. Although they could be maintained in the solution for 4 days and still be successfully transplanted, the interval before transplantation in this study did not exceed 15 min.
The skin of isogenic recipient rats was prepared by clipping, chemical depilation, and sterilization with 2% percative acid. A 2.0-cm incision was made on the dorsal midline between the shoulders, and s.c. pockets were prepared on either side by blunt dissection. A donor trachea with attached tubing to prevent curling of the transplant was inserted in each pocket, and the wound was closed with metal clips. Thus each recipient carried 2 transplants.

Twenty-eight days after transplanting, carcinogen pellets were inserted into the lumens through the cut end of the transplant, which was then ligated with a silk suture.

**Carcinogen Pellets.** DMBA powder (Eastman Kodak Co., Rochester, N. Y.) was melted at 122° and mixed with melted, laboratory grade white beeswax. The mixture was formed in a pellet maker (1) into cylindrical pellets 1.6 mm in diameter and 15 mm long, with a volume of approximately 30 μl and a weight of 24.45 ± 1.1 mg (S.D.). The concentrations of DMBA were adjusted on a weight basis so that the total amount of DMBA per pellet varied from 3200 to 10 μg (13.3 to 0.04% carcinogen). Control pellets consisted of beeswax without carcinogen.

**Carcinogen Assay.** The beeswax DMBA pellets were dissolved in 5 ml benzene and analyzed by UV absorbance at 300 nm (Beckman DB). DMBA concentration was determined by comparison with an absorbance graph of weighted standards (0.1 to 10 μg/ml). Beeswax alone dissolved in benzene had no absorbance at 300 nm and did not alter the absorbance of the DMBA standard.

**Experimental Design.** A total of 517 transplants were used. Groups of 12 to 58 transplants were exposed to pellets containing from 3200 to 10 μg DMBA. Control transplants were exposed to beeswax pellets without carcinogen.

Groups of transplants from each dose level along with control transplants were harvested at 9 time intervals, from 4 days to 8 months after pellet insertion. The pellets were removed and assayed to determine the amount of DMBA remaining. Regional lymph nodes and abdominal and thoracic viscera of the transplant-bearing rats were examined for tumor metastases.

Four groups of transplants with the 4 lowest carcinogen doses (10 to 210 μg) were used for a 22-month tumorigenesis study. The tracheas were palpated and measured twice a month to determine the time of appearance of tumors. The time when progressively enlarging tumors reached 1.0 cm was recorded, and when they reached 5.0 cm in greatest dimension the transplant and tumor were harvested and the diagnosis was confirmed microscopically. The tracheas remaining without palpable tumors after 22 months were harvested and examined histologically for the presence of lesions.

**Histopathology.** The transplants were fixed in Bouin's fluid. For uniform histological sampling, they were transected into 2-mm-long cylinders, embedded together on end in paraffin, and sectioned at 6 μm. In addition to hematoxylin and eosin, selected tissues were treated with Gomori trichrome, periodic acid-Schiff, Snook reticulum, or aldehyde fuchsin-Van Gieson stains and reagents.

**RESULTS**

**Determination of Carcinogen Dose and Dose Rate**

Assay of pellets for the amount of DMBA remaining after different time intervals in vivo provided a measure of dose and dose rate. The rate of carcinogen release was dependent on carcinogen concentration. For those pellets containing 325 μg or more, an early rapid release was followed by sustained release for from 2 to 6 months depending on the initial concentration (Chart 1). When plotted on a semilog scale (Chart 2), the carcinogen release for each concentration level could be described by a straight line with a relative
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S.D. of less than 10%. The average slope was 0.017. In this range of DMBA concentrations, the rate of release appeared to approach 1st order and was proportional to the amount of DMBA in the pellet. The amount of carcinogen delivered to the transplant, therefore, was approximately 1.7% per day of the amount remaining in the pellet. Thus the trachea was continuously exposed, but to progressively smaller amounts as time passed. When the initial amounts of carcinogen were 210 μg or less (0.8% and below), nearly all the carcinogen was released within 1 to 4 weeks (Chart 3).

Thus, although the dose rate was not constant, the amount of carcinogen delivered over any given period of time could be accurately determined.

Control Transplants

As previously reported (1), the mucosal epithelium of normal tracheas partially degenerated shortly after transplantation. When the blood supply was reestablished on the 4th day, there was a burst of mitotic and secretory activity, which subsided to base levels in 2 weeks. Four weeks after transplantation the tracheal transplants were lined by columnar mucociliary epithelium (Fig. 1). No significant changes occurred in the epithelium of control transplants exposed to beeswax alone. During the 22-month observation period, the lining epithelium gradually became low columnar and the mucus in the lumen was partially inspissated.

Serial Morphological Studies

3200- to 325-μg DMBA Pellets. At these carcinogen concentrations all the transplants exposed to DMBA underwent generalized squamous metaplasia followed in several months by squamous carcinoma. No significant difference could be attributed to the sex of the rats; therefore the data are combined.

At the earliest time point, 4 days after the start of carcinogen exposure, the columnar epithelium was elevated by basilar hyperplasia. After 1 week, the entire mucosal lining was uniformly replaced by squamous metaplasia with light cornification (Fig. 2). After 2 weeks the lumens of the transplants were filled with keratin and desquamated, cornified cells (Fig. 3). Severe squamous metaplasia of the mucosa and submucosal glands was characterized by hyperchromatic atypical basal and parabasal cells with numerous mitoses. Lymphoid cells were scattered through the adventitia and, to a lesser extent, the submucosa. One month after the start of carcinogen exposure, squamous keratinizing metaplasia persisted (Fig. 4) with, in addition, patchy myxoid degeneration of the adventitia and submucosa. Scattered lymphoid cells were numerous in the adventitia but did not form follicles. No plasma cells were found.

At 2 months of carcinogen exposure, all transplants at all dose levels exhibited downgrowths of atypical, keratinizing squamous epithelium that formed cystic tumors outside the confines of the original trachea. The lesions were diagnosed as invasive squamous carcinomas (Fig. 5). After 3 months, the transplants were greatly enlarged by invading squamous carcinoma. In those animals permitted to survive for long periods, local tumor growth progressed until some carcinomas exceeded 100 g (Fig. 6).

All 104 transplants harvested after 2 months or more of exposure to pellets containing 325 μg DMBA and above developed squamous carcinomas (Table 1). The neoplasms invaded the overlying dermis and underlying muscle as well as s.c. nerve sheaths, but metastases were not found. The stroma accompanying the carcinomas was often hypercellular, and in 20 instances fibrosarcomas or undifferentiated sarcomas were also found adjacent to the carcinomas. Sarcomas first appeared after 4 months of carcinogen exposure (Table 1) and were observed in the higher-dose groups. Because of the rapid replacement of the tracheal transplants by neoplasms, it was impossible to determine whether the tumors were multifocal. No predilection was observed for the laryngeal end of the transplant, as was previously reported (4) with different techniques.

The recipients' own tracheas were all normal histologically.

Table 1

<table>
<thead>
<tr>
<th>DMBA concentration/pellet</th>
<th>No. of transplants with carcinomas/no. of transplants tested at following mos. of exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg</td>
<td>%</td>
</tr>
<tr>
<td>3200</td>
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<tr>
<td>115</td>
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<td>40</td>
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<td>10</td>
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<tr>
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* Numbers in parentheses, number of transplants with sarcomas.

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210 to 10 \( \mu g \) DMBA. During the 1st 2 weeks, widespread hyperplastic-metaplastic changes occurred at the 210- and 115-\( \mu g \) dose levels. At 1 and 2 months metaplasias with severe cellular atypias developed, and the 1st invasive carcinomas appeared at 2 months (Table 1). Heterogeneous lesions ranging from atrophy to hyperplasia-metaplasia and occasional focal erosions occurred throughout the transplants. From 4 months multiple "preneoplastic" lesions (metaplasias with severe atypia and dysplasias), as well as microinvasive and invasive carcinomas, occurred.

At the 40- and 10-\( \mu g \) dose levels, the most common epithelial reaction during the 1st 4 weeks was hyperplasia, usually with preservation of the mucociliary characteristics. At later time intervals most transplants were difficult to distinguish from beeswax pellet controls. Only a few transplants in the 40-\( \mu g \) group showed conspicuous epithelial lesions such as small metaplastic plaques and occasional foci of dysplasia, the 1st being observed at 4 months. However, no frankly neoplastic lesions developed in any of the transplants during the 8-month observation period. The morphology of the various types of lesions was similar to that seen following benzo(a)pyrene exposure and is described and documented in greater detail in the accompanying paper (8).

Tumor Induction Study

Four groups of rats with 2 tracheal transplants each were used to study tumor development in the lower DMBA dose range from 210 to 10 \( \mu g \) over a 22-month period. The time of tumor onset was estimated by bimonthly palpation, and a tumor was assumed to exist when the palpated protuberance had reached 1 \( cm \) in diameter. Upon death of the animal (or upon sacrifice when the animal became moribund), the tumor was processed for histology to verify the macroscopic diagnosis. At 22 months the surviving animals were killed and their tracheal grafts were processed for histology. The results from this study are summarized in Table 2. The data show a decreasing tumor incidence and an increasing tumor latency with decreasing carcinogen dose. Three squamous cell carcinomas and 1 fibrosarcoma were detected in the 40-\( \mu g \) group. At the 10-\( \mu g \) level no carcinomas developed in 18 grafts within the 22-month observation period. The epithelial tumors observed in the study were squamous cell carcinomas with infrequent adenocarcinomatous admixtures. They were locally invasive, but distant metastasis was found in only 1 instance (115 \( \mu g \) DMBA after 8 months) to the lung. In several cases the rats died from the tumor that had developed in 1 of the 2 grafts with no tumor present in the other graft. The data thus are a low estimate of tumor incidence, since a malignancy might have developed in the 2nd transplant if the host had survived longer. Two of the transplants in the 40-\( \mu g \) group in which no tumors developed showed focal metaplastic and dysplastic lesions at 22 months. However, the epithelium of most of the transplants without tumors was unremarkable.

Fibrosarcomas developed in 16 out of 100 tracheal grafts (Table 2). Ten of them clearly had developed around the polyethylene tubing, including all the sarcomas in the control groups and the 10-\( \mu g \) DMBA group. The origin of the other 6 sarcomas was impossible to determine.

DISCUSSION

In previous studies (6) we established that carcinomas can be readily induced with carcinogenic polycyclic hydrocarbons in tracheas heterotopically transplanted to isogenic recipients. In those initial experiments 3-methylcholanthrene or benzo(a)pyrene crystals were incorporated into gelatin pellets, which were then inserted into established tracheal grafts. Although it was possible to induce tumors in this manner, we also found that this type of carcinogen delivery had drawbacks. Since the gelatin pellet dissolved within 10 \( min \) in the tracheal lumen, the carcinogen crystals were rapidly released and transported to the proximal end of the transplant, where most of the tumors subsequently developed. In later studies (1), to allow a more uniform exposure of the tracheal epithelium, carcinogens were incorporated into beeswax pellets, which do not dissolve in vivo. This was thought to have several advantages: a more even exposure of the tracheal epithelium (since the pellet fills most of the tracheal lumen and the carcinogen can make contact with the epithelium at the point of release from the pellet); slower, more steady release of the carci-
and better control over duration of exposure since the carcinogen-containing pellet can be removed at will. With these and other technical improvements (1) at hand, it seemed warranted to initiate systematic studies to obtain information on carcinogenic dose range (using polycyclic hydrocarbons known to be carcinogenic), tumor incidence, and tumor latency. We also wanted to obtain information on the amount of carcinogen released from the pellets at different time intervals (to estimate the dose of carcinogen delivered) and on the rate of release of the carcinogen from the beeswax pellets as a function of carcinogen concentration. This information was needed in order to interpret the tumor induction data and to design future studies of the effects of carcinogen dose rate on tumor induction. Beeswax pellets containing DMBA concentrations ranging from 13.3 to 0.04% were used. We found that the exposure duration and carcinogen dose rate was dependent on the starting concentration of DMBA in the beeswax pellets. Thus, with increasing concentrations, duration of exposure as well as cumulative carcinogen dose increases. We also found that the release rate for pellets with high and low carcinogen concentrations had different characteristics. Subsequently, we have discovered that the difference can be attributed to the limited solubility of DMBA in beeswax (B. Pal, R. Griesemer, and P. Nettesheim, unpublished data). The carcinogen dissolved in the wax carrier at concentrations of 0.8% (210 µg pellets) and below, while at higher concentrations the DMBA remained partially in crystalline form. Studies are presently underway to improve the carcinogen delivery system in this experimental model.

Different carcinogen concentrations were used to determine the effect of DMBA concentration (and dose; see above) on tumor latency, incidence, and type. With carcinogen concentrations of 325 µg and above, we found that the response of the tracheal transplants to DMBA was remarkably uniform. The degree of hyperplasia, metaplasia, keratinization, and cellular atypia in individual microscopic fields was representative of the entire trachea and of other tracheas sampled at the same time of exposure. In this range of DMBA concentrations, only minor differences were recognizable between groups, related mostly to the degree of edematous and inflammatory changes in the submucosa and adventitia and to a lesser extent to the height of the hyperplastic-metaplastic epithelium.

The 1st lesions that might be interpreted as microinvasive were noticed at 1 month. At 2 months of exposure, tumor nodules were present in all animals. Histological examination revealed invasive squamous cell carcinomas in all cases. At that time the doses delivered ranged from 280 to 910 µg DMBA. Typical dysplastic lesions and carcinomas in situ were not observed, possibly because of the rapidity with which the neoplasias develop at these highly carcinogenic dose levels. In some of the tracheal transplants, particularly at the higher DMBA dose levels, sarcomas developed together with the carcinomas.

With DMBA doses of 210 µg and below, development of lesions showed a great amount of variability, not only between different dose levels but also between different tracheas receiving the same carcinogen dose. Even within a given trachea, different types of epithelial changes occurred side by side. Preneoplastic and neoplastic lesions developed focally. The serial sacrifice studies as well as the tumorigenesis studies carried out over a 22-month period clearly indicate the dose dependency of tumor induction. They also suggest that the minimal tumorigenic DMBA dose in this system is somewhere between 40 and 10 µg.

The reasons for the low incidence of metastases are unknown. The squamous carcinomas varied in histological degrees of differentiation, but all were locally invasive. Four tumors selected at random were transplanted to isogenic nonimmunosuppressed hosts, and all 4 grew rapidly, killing the hosts in 3 to 4 weeks. Transplantable lines were readily established.

In conclusion, we have demonstrated that carcinomas can be induced by DMBA in the respiratory epithelium of grafted tracheas, that a dose-response relationship exists, and that the grafted trachea is sensitive to small amounts of DMBA. One limitation of the model system as described here is that the rate of delivery of carcinogen to the graft is not constant. We are, therefore, exploring the use of other vehicles and delivery systems to minimize this problem. Another complication is the appearance of sarcomas around the polyethylene tubing, although this is not unexpected in the rat; it suggests that carcinogenesis experiments in this model system may be restricted to 2 years. This problem can be avoided by sewing the grafts to the fascia rather than to polyethylene tubing (R. Griesemer and D. Martin, unpublished data).

REFERENCES

Fig. 1. Photomicrograph of a control trachea, 4 weeks after transplantation. The epithelium is mucociliary, and the lumen is filled with mucus. H & E, x 500.

Fig. 2. After 1 week of exposure to DMBA (delivered dose of 80 μg), the mucociliary epithelium is replaced by squamous metaplastic epithelium with basilar hyperbasophilia. H & E, x 585.

Fig. 3. Squamous metaplasia uniformly involves the entire mucosal lining and the submucosal glands. The lumen contains desquamated, cornified cells. Two weeks of carcinogen exposure (delivered dose of 180 μg). H & E, x 200.

Fig. 4. Keratinizing squamous metaplasia after 4 weeks of exposure to DMBA (delivered dose of 225 μg). H & E, x 313.

Fig. 5. Invasive squamous carcinomas (arrow) outside the original transplanted trachea (note surviving cartilage) after 2 months of carcinogen exposure with a delivered total dose of 280 μg DMBA. H & E, x 78.

Fig. 6. Large, s.c. ulcerated carcinoma at the site of a transplanted trachea exposed to DMBA for 6 months (delivered dose of 1240 μg).
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