Sustained Release of Benzo(a)pyrene from Silicone Polymer into the Tracheobronchial Tree of Hamsters and Dogs

Edwin C. Shors, Paul C. Fu, Kimito Matsumura, Arthur H. Cohen, and John R. Benfield

Department of Surgery, City of Hope National Medical Center, Duarte, California 91010 [E.C.S., K.M., J.R.B.], and the Department of Pathology, Harbor/UCLA Medical Center, Torrance, California 90509 [P.C.F., A.H.C.]

ABSTRACT

A method was developed to expose specific sites of the hamster and canine tracheobronchial tree to benzo(a)pyrene (BP) at quantitatively sustained rates. Implants for sustained release were formed by incorporating BP in a silicone rubber matrix at concentrations of 9.05 to 12%. Forty-nine hamsters and 12 dogs had a total of 86 implants surgically adhered to the tracheobronchial mucosa for up to 200 days. BP was released from the implants in hamsters and dogs as a first-order exponential function with a half-time of 54.8 and 44.5 days, respectively. Pathogenesis was progressively time and dose dependent. Squamous metaplasia with atypia regularly occurred in dogs within 150 days or after 7.17 mg BP and in hamsters after 50 days or 288 μg BP. Bronchogenic cancers occurred in 93% of our hamsters after 100 days and 467 μg BP. This method has applicability potentially as a bioassay for evaluating carcinogens in hamsters and currently as a means of producing a model of lung cancer in which neoplasia is induced at precise, selected sites.

INTRODUCTION

We have been seeking to develop experimental models of lung cancer in both large and small animals in which malignant tumors appear at localized and preselected sites with well-defined dose and time relationships. Such models will provide an opportunity to study the respiratory tract carcinogenesis phenomenon in a prospective manner.

The ability to experiment with lung cancer produced by chemical carcinogens was significantly enhanced when Saffiotti et al. (19) introduced a technique for producing a reliable animal model. In hamsters, he improved on the concept of serial tracheal instillations by attaching the carcinogen, BP, to a carrier, ferric oxide. His animals developed squamous cell carcinomas. Although the method of Saffiotti et al. is widely used, its disadvantages include the unpredictable location of the cancers, imprecise dose-response relationships, and the need for repeated instillations of carcinogen. Alternative techniques to circumvent these limitations include wire implants impregnated with carcinogen implanted in the lung parenchyma (1) or in the main-stem bronchi of rats (12). More recently, beeswax capsules laden with carcinogen have been introduced into s.c. tracheal homografts (15).

Much knowledge about carcinogenesis has been derived from experiments with small animals, but there is a need for a large animal model of bronchogenic carcinoma. With such a model, techniques for diagnosis and therapy, which are as yet inappropriate for patients and not applicable to small animals, could be evaluated. Our experience with recurrent submucosal i.b. injections of carcinogens in dogs has been reported recently (2), and we have described a method for implantation of silicone polymers which contain and gradually release carcinogen (13, 20).

The aims of this study were 2-fold: first, to define and compare the kinetics of carcinogen release from silicone rubber implants located in the tracheobronchial tree of hamsters and dogs; and second, to determine the carcinogenic potential of the approach. Our findings demonstrate that release rates were sustained and similar in both species, thus making reasonable the proposition that SRI have application to multiplespecies experimental carcinogenesis systems. The demonstration that cancers were produced in hamsters and that premalignant lesions with well-defined dose-response relationships were induced in dogs at preselected sites documents the genesis of 2 unique and supplementary models of experimental lung cancer.

MATERIALS AND METHODS

Animals. Fifty-four 8- to 10-week-old male Syrian golden hamsters (90 to 110 g) were housed 3 to 5 animals/cage (Simonsen Laboratory Animals, Gilroy, Calif.). Fifteen mongrel dogs (13 to 25 kg) of randomly selected sex and age were used. The animals were fed standard laboratory chow and water ad libitum.

Preparation of SRI. The matrix of the implants was a polymer of silicone (Silastic Elastomer 382; Dow Corning Corp., Midland, Mich.). This is a viscous liquid that can be homogeneously mixed with BP. Concentration of carcinogen up to 20% can be achieved. After BP (Aldrich Chemical Co., Milwaukee, Wis.) was ground into a fine powder (mean particle size, 5 μm), using a mortar and pestle, it was incorporated into the liquid polymer so as to constitute from 9.05 to 12.12% of the total weight. The slurry was solidified into a resilient solid matrix by the addition of 0.5% of the catalyst, stannous octoate. The solidification process lasted 10 to 15 min, and during this time the silicone BP aggregate was shaped into appropriate forms.

For hamsters, the implants were formed by drawing the viscous aggregate containing 10.5% BP into a glass tube (inside diameter, 1.5 mm) using a vacuum pump. Following solidification, the glass template was broken, and the matrix was cut into sections 2.5 to 4.5 mm long and 4.9 to 9.1 mg in weight. These implants contained 514 to 955 μg BP. A 4-0 stainless steel suture wire (Ethicon Corp., Sommerville, N. J.),
For dogs, the implants were formed by pouring the material into a glass Petri dish (13 x 60 mm) to a depth of 1.5 mm. The implants contained either 9.05 or 12.12% BP. Following polymerization, plates of BP polymer were constructed. Initially, we used squares, but consistency of size was substantially improved by punching out discs of polymer with a cork borer 6 mm in diameter. The BP polymers implanted into dogs varied from 20.5 to 206.3 mg, and they contained 1.85 to 18.67 mg BP.

**Implant Placement and Retrieval.** Hamsters were anesthetized with i.p. sodium pentobarbital (100 mg/kg). A tracheotomy was done, and the implant with the hook end pointing rostrally was quickly but carefully inserted (Chart 1) into the trachea using a microbiopsy forcep (Machid America, Inc., New York, N. Y.). The tracheotomy was closed by using a 4-0 interrupted suture. The condition of the hamsters was monitored twice daily. If a hamster died, the implant was recovered at autopsy. Hamsters which did not die were sacrificed by renal artery exsanguination. The implant retrieval schedule was 4 per week for the first month, 4 per fortnight for the next 2 months, and 4 per month thereafter. The hearts and lungs were removed en bloc. The implants were located and retrieved by a longitudinal incision of the tracheobronchial tree. The bronchial tissue and accompanying lung parenchyma were fixed in 10% neutral buffer formalin solution. The histology of the tissue adjacent to the implants was evaluated in all specimens, and serial sectioning of the tracheobronchial tree was performed on selected specimens.

The technique for implantation in dogs has been described previously in detail (13). Briefly, the implants in the right mainstem bronchus or adjacent trachea were either secured into a submucosal pocket between the epithelium and the cartilaginous rings or fixed to the epithelial surface with suture (Chart 1). Three to 4 implants were placed in each dog at least 2.0 cm apart. Capsules were randomly retrieved at a rate of 3 per week for the first month and approximately one per week thereafter by endobronchial excision using a universal biopsy forcep (Pilling Company, Fort Washington, Pa.). Serial biopsies of the mucosa adjacent to the implants were done at monthly intervals via bronchoscopy.

**Quantitation of BP.** All capsules were stored in the freezer until chemical analysis was performed as a group. The method for determining the rate of release of BP from the silicone polymer was based on measuring the residual amount of BP in the capsule. Since BP is highly soluble in acetone, recovery of residual BP was accomplished by placing the capsules 3 times in 5 ml of acetone for 24 hr. The acetone washings were combined and evaporated under nitrogen. Dilutions of 1:10 and 1:100 were made, and the aliquots of BP were dissolved in 10 ml of heptane. The UV absorbance of the samples, standards, and controls were determined at 296 nm in a spectrophotometer (Model DU; Beckman Instruments, Palo Alto, Calif.). Recovery of BP from the implants was determined to be >98%, and the coefficient of variation from the analytical technique was 3.35%. The amount that had been released was calculated by subtracting the amount remaining from that originally present.

**Controls.** Implants of silicone rubber without BP were prepared. They were constructed and surgically inserted as described previously. The hamsters in the control group were all sacrificed after 200 days, and the implants from dogs were harvested according to the schedule described for implants which contained BP.

**Statistical Analysis.** Rates of BP release and release constants were determined by deriving a ratio of the amount of carcinogen retained in the capsule after implantation to the amount of carcinogen originally contained. This ratio was expressed as a percentage. Statistically, the logarithm of percentage was related to the time over which the release occurred by a least-squares regression curve. In addition, a regression curve for the logarithm of the percentage remaining was constructed with the constraint that the curve pass through 100% at Day 0 (21).

**RESULTS**

**Effects of Silicone Rubber.** Twenty-five hamsters had implants without carcinogen, and 3 dogs had 9 control implants. No significant histological changes were induced in either the hamsters or dogs. Specifically, the epithelium adjacent to the control implants was normal in 6 hamsters and underwent columnar hyperplasia in 5 hamsters, basal hyperplasia in 12 hamsters, mild squamous metaplasia in one hamster, and ulceration in 2 hamsters. Inflammation of the bronchus was present in 16 hamsters.

**Kinetics of Release in Hamsters.** Forty-nine hamsters had SRI. There were no operative or immediate postoperative deaths. There were 6 premature deaths from tracheobronchial obstruction 2 to 42 days postimplantation. Four of these were from tracheal obstruction by the SRI, and 2 were from displacement of the SRI into the proximal mainstem bronchus. Four hamsters were cannibalized.

Of the 49 SRI, 4 were recovered in the trachea and 30 were found in the right lung bronchi almost invariably at or near the same site. Six SRI were in the left lung, 2 were in the mainstem bronchus, and 4 were in the lower lobe. Nine SRI either could...
not be found at autopsy or were lost when hamsters were cannibalized. Forty SRI were judged worthy of chemical analysis.

The amount of BP remaining in the capsule was expressed as the logarithm of the percentage of the amount originally present. This was related to the time over which the release occurred, i.e., the time from implantation. This relationship was derived from 40 observations and was linear \( p < 0.05 \), implying a first-order exponential release rate. The means ± S.E. at each interval as well as the least-squares regression line are presented in Chart 2a. The slope of the line was 0.00529 and the y intercept was 94.8%. The release constant, \( \lambda \), was 1.21%/day \( (\lambda = \text{slope} \times 2.303) \).

When the least-squares regression line was constrained to pass through 100%, the release constant was 1.27%/day. To describe more clearly the kinetics of release, the data were converted to percentage of BP released and plotted as an exponential function on linear coordinates (Chart 2b). In this form, the curve was constrained to pass through the origin. The release half-life, \( t_{1/2} \), analogous to that utilized in describing radiation decay or bacterial growth, was 54.8 days.

The data also adhered to a simple regression curve with a slope of 0.342, e.g., 0.34% released per day \( (p < 0.05) \), and an intercept of 22.8%. When the linear regression curve was forced through the origin, the slope was 0.615.

Kinetics of Release in Dogs. The release rate function for

---

**Chart 2.** a. percentage of BP remaining in capsules as a function of the time since implantation in hamsters. The mean value at each interval and unconstrained least-squares regression line are presented; bars, S.E. A release constant \( \lambda \) of 1.21%/day was generated. b. The theoretical percentage of BP released from the capsule as a function of time. The curve and its 95% confidence interval was constrained to pass through the origin. This curve reflects a release half-time of 54.8 days.
were found in 13 of 14 hamsters. Ten of the cancers were to 2-week period of ulceration or normal respiratory epithelium.

Bronchoscopic findings in the hamster bronchi adjacent to the implants.

For the next 10 weeks or after 400 &g BP were released, the squamous metaplasia was observed. This was followed by a 1-

During the first week after 54 &g BP had been released, mild

during the retrieval location could be a source of variability. However,

The matrix of our implants is an inert polymer of silicone.

The results of biopsies taken from dogs still alive and under study have been reported previously (13). Briefly, squamous metaplasia was evident within 1 week of implantation. Basal hyperplasia was frequently associated with the squamous metaplasia overlying the submucosally placed pocket implants, whereas squamous metaplasia without hyperplasia was found adjacent to the mucosally fixed implants. These epithelial changes persisted and were frequently associated with atypical cellular morphology and increased mitotic activity by 16 to 20 weeks. To date, only these preneoplastic changes have resulted in our dogs from SRI of BP implanted into the tracheobronchial tree.

DISCUSSION

A lung cancer model in which cancer occurs at a preselected and localized site would be an invaluable tool for both basic and clinical research. We are seeking to develop this model in hamsters and dogs. It is clear that canine lung carcinogenesis is a long-term project (2), although in hamsters lung cancers form readily (19, 20). Each animal model has advantages and shortcomings, and therefore they are supplementary research methods. The success we had in using SRI to cause lung cancers in hamsters encourages us to pursue the approach further in dogs.

Our initial approach in dogs utilized recurrent injections and applications of carcinogens into the bronchial mucosa (16). This allowed precise localization of the carcinogens and opportunity for regular evaluation by serial biopsies, but the investment of time by skilled personnel was inordinately high.

It has been shown in small animals that there is a rapid clearance (<24 hr) of materials injected into the trachea (12). We know of no such data for dogs, but it is reasonable to assume that chronic exposure of the epithelium to carcinogens in dogs requires an implanted or recurrent source. The SRI approach is a step toward the reliable production of experimental lung cancers with a single manipulation of the animal followed by an undisturbed, well-described latency period during which the cancer grows to a desired stage.

The matrix of our implants is an inert polymer of silicone. This material has proven to be effective for the in vivo release of cardioactive drugs (5), steroids (10), and antidiabetic medications (6). We have found that it can be readily molded into almost any size and shape. In addition, it can be formulated with polycyclic aromatic hydrocarbon carcinogens up to a concentration of 20%. The shape of the release curve, its variability, and its interpretation merit discussion. The release rate from this type of implant theoretically conforms to Fick's Law of Diffusion. This states that the release rate is linearly proportional to the surface area of the capsule in contact with tissue and to the concentration gradient between the capsule and tissue. Therefore, release rates expressed as a percentage of material available for release are affected by the surface-to-weight ratio. In our preparation, the cylinder diameters of the hamster implants and sheet thickness of the dog implants were constant. Therefore, implant weight was theoretically proportional to releasing contact with tissue, and thus there was variability of active surface area.

Pooling of all the recovered hamster implants regardless of the retrieval location could be a source of variability. However, a statistical analysis using only the 30 implants recovered in the right lung of hamsters did not significantly alter the kinetics curve or its variability. The coefficient of variation for the quantitative analysis of BP reflects the homogeneity of the carcinogen-polymer complex as well as the reliability of the analytical recovery procedure. Since the coefficient of variation

### Table 1

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Dose (µg BP released)</th>
<th>Squamous metaplasia</th>
<th>Dysplasia</th>
<th>Carcinoma in situ</th>
<th>Carcinoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–24</td>
<td>103 ± 74</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2 SC</td>
</tr>
<tr>
<td>25–49</td>
<td>267 ± 79</td>
<td>4</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50–99</td>
<td>355 ± 152</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>1 PD</td>
</tr>
<tr>
<td>100–149</td>
<td>625 ± 118</td>
<td>1</td>
<td>3</td>
<td>3 SC</td>
<td>2 SC</td>
</tr>
<tr>
<td>150–199</td>
<td>682 ± 125</td>
<td>1</td>
<td>3</td>
<td></td>
<td>1 PD</td>
</tr>
</tbody>
</table>

* Mean ± S.D.

SC: squamous cell carcinoma; PD, poorly differentiated carcinoma; A, adenocarcinoma; Mixed, mixed squamous cell and adenocarcinoma.
was low (3.35%), it appeared to us that the variance we found within the regression was related to biological rather than to analytical variability.

One potential biological variable affecting both the surface area and the concentration gradient was encapsulation due to an inflammatory reaction. However, this did not appear to have occurred either at a gross or microscopic level. In fact, all the implants that were located were removed easily from the bronchial wall. Another variable affecting the rate of release was the concentration gradient between the capsule and the tissue. With our approach, this gradient was constantly decreasing because the concentration of carcinogen in the capsule decreased over time, while presumably the concentration in the tissues increased. This assumes that the metabolism rate was less than the release rate and that the tissue sink was not infinite. These facts and assumptions result in an exponential release rate versus time function. That is, the absolute amount of carcinogen released decreased over time. As a consequence, our approach can only loosely be defined as a sustained release. The release kinetics for both our hamster and dog models, however, also conform to a linear function. Although this is contrary to theoretical considerations, release functions with zero order kinetics are optimal for releasing carcinogens at a constant rate (10).

Two theoretical approaches for maintaining constant release of a chemical, i.e., rigidly defined sustained release, have been described by Kinel and Rudel (10). One is to fill tubing, e.g., silicone tubing, with the chemical and seal the ends. If the chemical is silicone soluble, it will be released at a constant rate because the surface area and concentration remain constant. The limitations of this method are theoretical and technical. The theoretical problem is that the tissue concentration surrounding the implant will likely increase over time, thereby progressively decreasing the concentration gradient. The technical limitations are that the size of the capsule would need to be large and that the chemical would need to have an acceptable silicone permeation constant. The second method for maintaining constant release rate is to compose the implant matrix out of a substance that is biodegradable and absorbed at the same rate as the drug. The theoretical problem with this approach is that a constantly decreasing surface area causes a decrease in the release. For these reasons, the SRI approach we have used is as satisfactory as the alternatives.

Although in vitro models for evaluating release kinetics have been developed (18, 22), their ability to correlate with in vivo studies has been less than satisfactory (17). Apparently, biological variability significantly affects release rates. In vitro models can, of course, serve as screening methods to test the effects of concentration and types of carcinogens on release rate, but the ultimate test of a carcinogen delivery system is the production of cancers at preselected sites of the tracheobronchial tree.

A requirement of an ideal carcinogenesis animal model is that it localizes the developing cancers to a predictable site. One of the first successful models of respiratory carcinogenesis in small animals was predicated on depositing and confining the carcinogen to the tracheobronchial mucosa (19). This was achieved by attaching BP to the carrier molecule, ferric oxide. More recently, carcinogen dissolved in gelatin has been shown to cause selectively peripheral lung cancers when instilled into the tracheobronchial tree (9). These approaches, nevertheless, require repeated manipulations and result in imprecise localization of the cancers. To mitigate these problems, the sustained-release approach for carcinogen administration was developed over 40 years ago (1). Laskin et al. (12) expanded and improved on these early methods by implanting in rat bronchi a wire mesh impregnated with polycyclic aromatic carcinogens. Although squamous cell cancers were produced and sigmoidal dose-response relationships were demonstrated using the method, the kinetics of release were not rigorously defined.

Other matrices for release of carcinogen into either the urinary bladder, s.c. tissue, or tracheobronchial tree have been described (3, 7, 8, 14, 15, 17, 18, 22, 23). These have consisted of agar (11), gelatin (9), urethane fiber (14), paraffin, such as beeswax (7, 15), and steroid, such as cholesterol (3). Combinations of these materials have also been used (17, 23).

Hirano et al. (8) induced epidermoid cancers in rats by injecting molten beeswax and 3-methylcholanthrene into the tracheobronchial tree. This, however, is a difficult and traumatic technique and does not lend itself to accurate dose-rate and dose-response relationships.

The rat heterotopic tracheal graft model has been promulgated as a method for testing different chemicals as vehicles for the sustained release of carcinogens (7, 14, 17). Using this model, it was shown that 7,12-dimethylbenz(a)anthracene could be released from beeswax as a first-order exponential with a half-time of about 40 days (7). This was very similar to the half-time we found in our model when BP was released from silicone rubber. However, when using the rat tracheal graft system to release BP from beeswax, Nettesheim et al. (15) demonstrated an exponential half-time release of only 25 days. Nevertheless, the histogenesis of the 2 models was similar. Following an immediate period of hyperplasia, ulceration and atrophy were observed. This was followed for several months by squamous metaplasia and dysplasia culminating in in situ and invasive squamous cell cancers. In both models, occasional mixed squamous and adencarcinomas were observed. However, the rate of carcinogenesis was higher and the dose of carcinogen was lower using our preparation in hamsters. Thirteen of 14 of our hamsters which had implants for more than 100 days and had received less than 800 μg BP had cancers, and only 10 of 20 of the rat tracheal grafts developed cancers, although they received more than 900 μg BP for over 180 days. Comparison of our results, however, must be tempered with the recognition of species differences.

It is premature to be expansive about canine carcinogenesis with SRI, but it would be incomplete not to include our early findings in dogs. We have not yet produced lung cancers in dogs with SRI containing BP in the tracheobronchial tree. In carcinogenesis studies using other administration techniques but the same carcinogen, we have, however, shown that squamous cell cancers appeared in 8 dogs from 20 to 56 months after the initial exposure to BP. After multiple injections or applications of BP, cancers were always preceded by dysplasia that was grossly and histologically indistinguishable from the dysplasia found after 4 months of SRI. Sufficient time has probably not elapsed for cancers to appear in these dogs, but we are confident that they will. This belief is supported by a

---

pilot study in which a carcinosarcoma was induced by SRI containing 7,12-dimethylbenz(a)anthracene in the lung parenchyma of a mongrel dog within 8 months. The inclusion in this report of the studies on dogs is intended primarily to corroborate the release kinetics found in hamsters and to demonstrate the potential of the SRI approach in multispecies systems. Our approach to the use of carcinogen-laden silicone polymer has advantages over preexisting sustained-release techniques. These include the fact that the polymer can be located and is well tolerated in the intact tracheobronchial tree or lung parenchyma rather than in a tracheal graft. It is, therefore, a more physiological preparation with intact lymphatic function and free flow of mucous. In addition, the carcinogen can be localized to affect specific tissues, such as bronchial, tracheal, or even alveolar tissues. Undoubtedly, this approach would be equally effective for carcinogenesis models of other organs. Moreover, the amount of carcinogen remaining in the capsule can be precisely determined because of the inert nature of the silicone rubber matrix. In the dog model, we have shown that exposure to carcinogen can be abruptly terminated by endobronchial excision. Most important, we have shown that this approach is suitable for both large and small animal models of lung cancer and is not confined to a particular species.

Certainly, there are aspects of our experiments that need to be defined further. For instance, we do not know the ultimate fate of the carcinogen or how much of it is lost to metabolism, to the Airways, to the pulmonary macrophages, and to the lymphatic and blood circulation. We also do not know how other carcinogens would respond to the method. To date, it is clear that bronchogenic cancers can be induced by the sustained release of BP from silicone rubber.

ACKNOWLEDGMENTS

We thank Dr. Joan Fu for her invaluable guidance in the development of the methodology and Thomas Jensen for his superior technical assistance.

REFERENCES

1. Andervont, H. B. Pulmonary tumors in mice. IV. Lung tumors induced by subcutaneous injection of 1,2,5,6-dibenzanthracene in different media and by its direct contact with lung tissues. Public Health Rep., 52: 1584–1589, 1937.

Fig. 1. a, portion of bronchial mucosa showing severely atypical squamous epithelium. The changes are so extensive in places and involve the entire thickness of the mucous membrane that they approach a carcinoma in situ state. H & E, × 200. b, well-differentiated invasive squamous cell carcinoma. There are extensions of malignant cells extending deep into the underlying stroma. H & E, × 100. c, adenocarcinoma, composed of sheets of cells (lower portion) and well-defined glandular structures. There are inflammatory cells present because of adjacent necrosis of the tumor. H & E, × 200. d, poorly differentiated carcinoma. This carcinoma is composed exclusively of spindle-shaped cells; no squamous differentiation or features of specific mesenchymal cells could be identified. It is equally possible that this represents a poorly differentiated squamous cell carcinoma or a tumor derived from soft tissue elements of the bronchial wall. H & E, × 200.
Sustained Release of Benzo(a)pyrene from Silicone Polymer into the Tracheobronchial Tree of Hamsters and Dogs

Edwin C. Shors, Paul C. Fu, Kimito Matsumura, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/40/7/2288

**E-mail alerts**  Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.