Journal of Cellular Biochemistry, Supplement 17F:104–117 (1993)

# Hamster Bronchial Carcinogenesis Induced by Carcinogen-containing Sustained Release Implants Placed Endobronchially: A Clinically Relevant Model

**William G. Hammond, MD and John R. Benfield, MD**

Division of Cardiothoracic Surgery, Department of Surgery, School of Medicine, University of California Davis, Sacramento, CA 95817

**Abstract** For several widely appreciated reasons, the Syrian hamster has been the most frequently used experimental animal in investigations of conducting airway carcinogenesis. To develop a model where bronchogenic cancer arises focally at a predetermined site, we used the Laskin-Kuschner self-retaining intrabronchial pellet principle, employing a carcinogen-containing silastic polymer sustained release implant (SRI). The SRI is placed in the right lower lobe bronchus via tracheostomy; when modified, the SRI can be removed without loss of the animal. Special preparation of the SRI implant site after fixation but prior to paraffin embedding allows for full histopathological examination of the carcinogen-affected target tissue. Logistic regression analysis of histological findings provides valid quantitative inter-regimen comparisons of histomorphic classifications suitable for determining modulation of carcinogenesis by external influences.

Using this model, we demonstrated that the sequential progression of carcinogenesis (SPC) in hamster bronchus is similar to that which occurs in humans and in dogs, including both the ploidy increases that are progressive during the SPC, and the histological patterns of the induced cancers. We have shown genetic variation in susceptibility to carcinogenesis among inbred hamster strains, and have assessed effects of Bacillus Calmette-Guerin (BCG) immunostimulation on the SPC. Time/dose response studies were performed, as were comparisons between four polycyclic aromatic hydrocarbon carcinogens. Systemic administration of 5-azacytidine (AZC) soon after SRI placement was found to inhibit the SPC, to alter the ploidy changes during the SPC and in the eventual cancers, and to affect the degree of differentiation of the cancers. Studies using removable SRIs have assessed the duration of carcinogen exposure required to induce a neoplastic transformation that proceeds to cancer without further carcinogen exposure. Serial syngeneic transplantation of cancers arising in inbred animals has shown that the degree of tumor differentiation is affected by the extent of host immunocompetence, and has also led to development of models for study of the processes of metastasis. 

**Key words:** hamster lung cancer, bronchial carcinogenesis model

The first major international conference concerning lung tumors in animals was held in Perugia, Italy, in 1965. At that meeting, Rowe [1] proposed a set of criteria to define the optimal animal model for experimental studies of lung tumors; he also believed that results obtained from this model would be relevant to an improved understanding of lung cancer in hu-

Address correspondence to William G. Hammond, MD, Thoracic Research Laboratory, ARS, Bldg J-1, University of California Davis, Davis, CA 95616.

mans. The criteria include the following: (1) the animal should be small enough that large numbers can be used for statistical validity; (2) the natural lifespan of the animal should be short enough so that neoplasms can be induced in a timely fashion; (3) the species should have a low incidence of "spontaneous" lung cancer; (4) there should be no interference from other types of respiratory disease; (5) carcinogens should be delivered via the conducting airways, as if by inhalation; and (6) the histopathological types of the resulting tumors should be the same as those in humans. We have added the following: (7) the sequence of preneoplastic stages should be the same as that occurring in humans and should be induced by chemicals believed responsive for human lung cancer; (8) the sequence and its time-dose relationships should be reproducible; and (9) the carcinogen-induced changes should occur at a known location, with a latency period sufficient to allow serial observations but short enough to be practical. This report describes the small animal lung cancer model we have developed, and presents the findings obtained during its use related to the criteria set forth above.

## THE SPECIES SELECTED

The Syrian or Golden hamster (*Mesocricetus auratus*) was selected for a number of reasons, many of which are well known. The species is a small, hardy animal, readily available so that large numbers of animals can be used for valid statistical analyses. Its natural lifespan is less than two years, it has a very low incidence of respiratory infections, the extent of respiratory diseases which might interfere with carcinogenesis studies is insignificant, and the incidence of "spontaneous" lung neoplasms is negligible. Because it has been widely used as a research animal, its husbandry and dietary requirements are well defined. Not as widely known is the fact that inbred (syngeneic) strains of Syrian hamster are available.

Lastly, although outbred hamsters were used almost exclusively in earlier work, the hamster has been the experimental animal used most often for investigations of chemical carcinogenesis of the respiratory tract. This provides a substantial background of information upon which to base additional investigations.

## THE SUSTAINED RELEASE IMPLANT

Preparation of sustained release implants (SRIs) and their placement have been previously described in detail [2]. Briefly, carcinogen is emulsified in liquid silastic polymer at the desired concentration, a solidifying catalyst is added, and the still-liquid mixture aspirated into thin-wall glass tubing of the desired internal diameter. Following solidification, the carcinogen-containing silastic rod is cut into 3.5 mm segments; a fine steel wire is inserted and bent over to form a retaining hook. The SRI is placed in the right lower lobe bronchus via tracheostomy; if desired, a fine monofilament suture is placed through the SRI for later removal without loss of the animal.

Significant aspects of the SRI method for delivering chemical carcinogen to the bronchial epithelium include the following:

1. From its endobronchial site of placement, carcinogen released from the SRI reaches the target bronchial epithelium from within the conducting airway, as do inhaled substances.
2. The silicone polymer material can be shaped into any desired form, and is biologically inert. In our first study, 20 carcinogen-free SRIs were left in place for 200 days, with no preneoplastic changes noted in the epithelium at the implant site.
3. By relatively simple methods, SRIs retrieved at autopsy can be readily analyzed to determine the amount of carcinogen remaining in the SRI. The extraction procedure recovery rate is >98%, with a coefficient of variation of 3.35%. From such determinations, the release rate of carcinogen from the SRI can be determined and time-dose responses calculated.
4. Carcinogen release rates from SRIs of the same composition have been shown to be the same, whether the SRIs were placed in hamsters or dogs. This indicates the absence of species specificity for carcinogen release rates and confirms that release rates are functions of the concentration and type of carcinogen employed.
5. Continuous release of carcinogen from

the SRI ends at about the same time after placement as the earliest microscopic cancers appear in the epithelium at an SRI site. Thus the carcinogenic process proceeds through an essentially undisturbed latency period. This duration of carcinogen exposure is also about the same as that which occurs in the Saffiotti model [3], providing a useful parameter to compare results from the two methods.

6. Only a single manipulation of the animal is required, and the epithelial changes resulting from SRI placement occur at a known site in the lung, both factors that provide useful logistic simplification.
7. Our initial study with this approach used benzo(*a*)pyrene [B(*a*)P] because it was used in most earlier hamster respiratory carcinogenesis studies. The SRI induced cancers in 100% (13/13) of animals sacrificed at 100 or more days following SRI placement. Animals sacrificed earlier showed typical preneoplastic alterations in the epithelium at the SRI site; the severity of change was dependent on the duration of carcinogen exposure.

## DIFFERENT CARCINOGENS IN DIFFERENT AMOUNTS

During an expanded study, SRIs containing either 10% or 2% B(*a*)P or 10% or 2% methylcholanthrene (MCA) were placed in a total of 369 hamsters. Groups of 5–10 hamsters were sacrificed at progressively increasing intervals beginning at one week after SRI placement.

Carcinogen release rates were determined for each of the four varieties of SRI. The findings, shown in Figure 1, indicated a single-order exponential function common to all four varieties of SRI. The half-time for B(*a*)P release was slightly longer (~40 days) than that for MCA (~32–35 days).

Although carcinogenesis in respiratory epithelium is a progressively continuous process in humans [4] and dogs [5], as well as in hamsters [6,7], 11 histopathologic diagnoses within four broad categories were utilized to define stages of the SPC to facilitate comparisons among regimens. The broad categories included: (1) normal or reactive changes, including early nonspecific alterations such as inflammation and basal or columnar hyperplasia; (2) squamous metaplasia, ranging in severity over five steps from focal regular squamous metaplasia to severe atypia; (3) cancer detectable only by microscopic examination, *i.e.*, carcinoma *in situ* and microinvasive carcinoma; and (4) grossly apparent and histologically confirmed invasive cancer of three size ranges. The histologic criteria used for diagnostic subdivisions within the broad categories were those previously described [2]; diffuse squamous metaplasia was defined as characteristic mucosal change that either involved at least 1/4 of the bronchial circumference or extended at least 400 $\mu$m along the bronchial axis. The categories of grossly apparent cancer were minimal, <3 mm diameter; medium, 3–10 mm diameter; and extensive, >10 mm diameter. These serial stages of the SPC were present in all four groups of hamsters; however, the time course of development was significantly prolonged in hamsters receiving 2% SRIs compared to those receiving 10% SRIs. The differences between carcinogens at the same dose were minimal [8]. Time-dose response data for the four varieties of SRI are presented in Table I.

A degree of variability, similar in all groups, is most likely related to the variation in post-transformational proliferation rates of clonal cell populations after the progenitor differentiates from one stage of the SPC to the next. The somewhat greater variability seen from 2% SRIs may arise from differential susceptibility to carcinogenesis that is inherent in an outbred animal population and becomes more manifest under the influence of a less vigorous carcinogenic stimulus.

Two other varieties of polycyclic aromatic hydrocarbon carcinogens were studied in the same fashion. 7,12-Dimethylbenz(*a*)anthracene (DMBA) was used in SRIs at 2, 5, and 10% concentrations because of the extensive use of this compound in a different form of SRI by Nettesheim and his collaborators [9]. 7-Chloromethylbenz(*a*)anthracene (CMBA) was employed because Peck *et al.* [10] found that this derivative was more effective and less toxic as a respiratory carcinogen than DMBA. Our findings are shown in Table II.

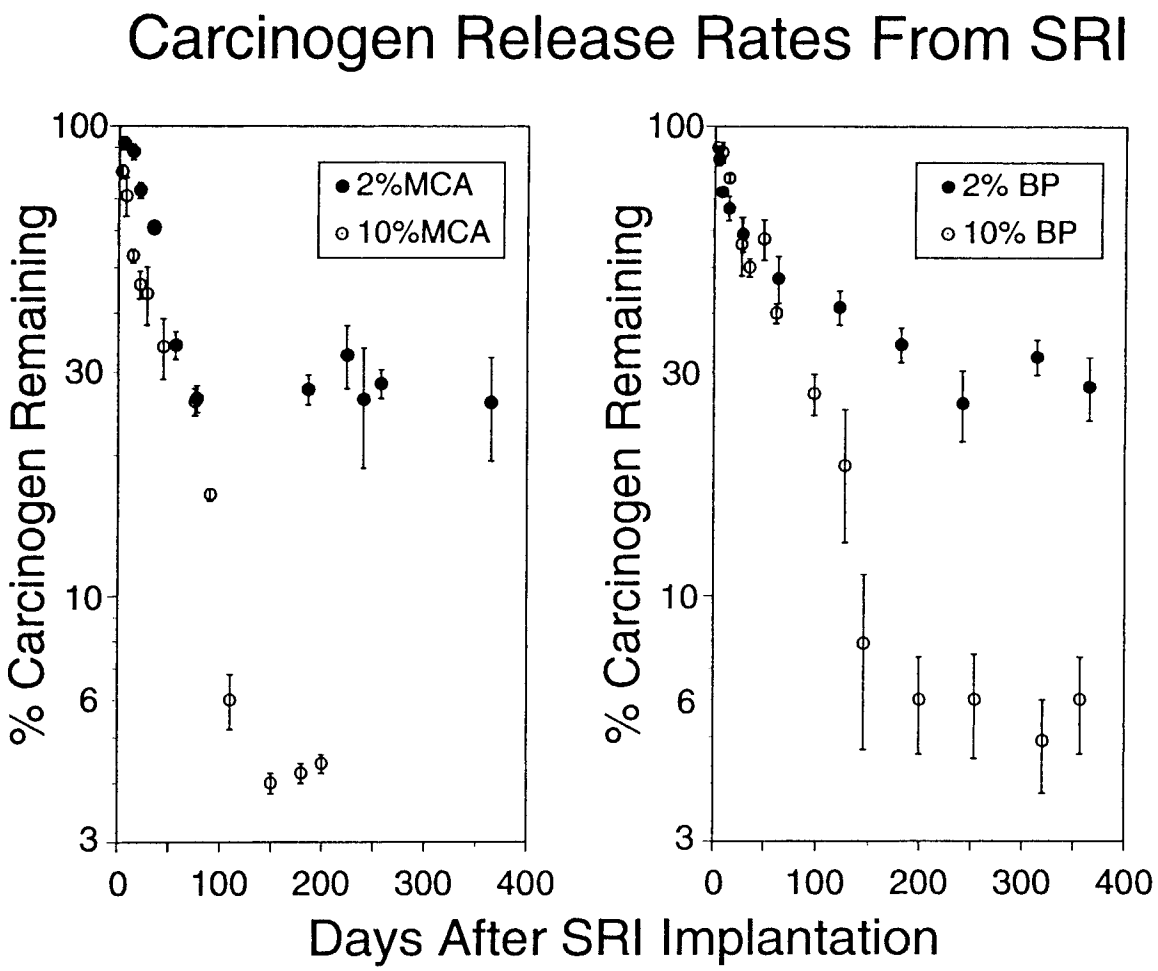


**Fig. 1.** Semi-logarithmic plots of carcinogen release rates from endobronchially placed SRIs in hamster, showing typical first order exponential configuration. Note similarity between 2%- and 10%-containing SRIs for both carcinogens. Note also cessation of carcinogen release at ca. 30% for 2% SRIs and ca. 6% for 10% SRIs; these indicate the same amounts of carcinogen (0.042 mg) remaining in the SRIs and may reflect either the lower limit of sensitivity of the analytic method or that amount of carcinogen within the SRIs below which concentration gradients do not develop to shift carcinogen out of the SRI.

Although DMBA toxicity appeared tolerable when used in Nettesheim's heterotopic tracheal transplant model [9], when it was used in SRIs in the intact orthotopic lung, it proved unacceptably toxic; similar findings were noted when DMBA was instilled at intervals directly into the trachea instead of placed in SRIs [11]. Further, DMBA was not a very satisfactory carcinogen; the combination of excess toxicity and suboptimal cancer incidence led us to discontinue its use [12]. On the other hand, even at 5% in the SRI, CMBA proved to be an effective carcinogen with minimal toxicity. We have not employed it further for two reasons: it is not readily available, and it is not known whether this compound is a constituent of tobacco smoke.

Because of recent reports demonstrating the marked effectiveness of systemically administered 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in producing lung cancers, and because this compound and its precursors are the primary nitrosamine carcinogens in tobacco smoke, we explored its use by topical administration in SRIs containing 10% NNK. No epithelial abnormality whatsoever was seen

TABLE I. Time-Dose-Response Highlights

| Carcinogen | % Carcinogen in SRIs | Duration SRI Exposure | No. With Cancer (total no.) | mg Carcinogen Released From SRI | No. With Cancer (total no.) |
|---|---|---|---|---|---|
| B(*a*)P | 2 | >170d | 12/31 (39)* | 0.23 | 11/17 (65) |
| B(*a*)P | 10 | >90d | 56/67 (84) | 0.50 | 55/68 (81) |
| MCA | 2 | >170d | 7/23 (30) | 0.21 | 6/15 (40) |
| MCA | 10 | >100d | 12/14 (88) | 0.63 | 12/15 (80) |

* Parenthetic nos. = percentages

TABLE II. Comparison Between Two Polycyclic Aromatic Hydrocarbon Carcinogens

| Carcinogen | % Carcinogen in SRI | No. of Hamsters | % Attrition Before Risk | % Incidence Cancer—At Risk |
|---|---|---|---|---|
| CMBA | 5 | 22 | 5 | 70 |
| DMBA | 2 | 42 | 45 | 13 |
| DMBA | 5 | 23 | – | 17 |
| DMBA | 10 | 63 | 58 | 9 |

at the SRI site in animals sacrificed 2–10 weeks following SRI placement. Because NNK is much more water-soluble than are the polycyclic aromatic hydrocarbons, it seems likely that the release of NNK from the SRI and its subsequent clearance from the endobronchial site was so rapid that the duration of carcinogen exposure was insufficient to induce significant epithelial abnormalities.

In canine models, we described a reproducible, focally originating SPC that has led to epidermoid cancers [5]. Changes in total cellular DNA content (QDNA) occurring in the hamster model during chemically induced bronchial epidermoid carcinogenesis have been compared with the changes found in human patients with suspected or known pulmonary neoplasms, high-risk coke oven workers, and in the canine model [13].

Cytologic preparations were made from sputum, bronchial washings, or from touch preparations of bronchial epithelium or resected cancers. After cytodiagnosis by the Papanicolaou method, specimens were de-stained and re-stained with the Feulgen stain. QDNA was measured by image analysis cytophotometry. In contrast to flow cytometry, image analysis provides direct correlation between progressive cellular abnormality and QDNA in individual cells.

These findings (Table III) indicate a progressively increasing hyperploidy accompanying the progressively increasing severity of mucosal abnormality occurring with the SPC. Note that severely atypical squamous metaplasia is supratetraploid in all three species. The QDNA SPC relationship was the same for all carcinogens which had mucosal effects.

## DIFFERENT HAMSTERS

Our earlier work and all previous studies of bronchial carcinogenesis from topically applied chemical carcinogens in Syrian hamsters used

**TABLE III. Quantitative Total Cellular DNA During Epidermoid Bronchial Carcinogenesis in Three Species**

| SPC Stage | Hamsters (n = 72) | Humans (n = 53) | Dogs (n = 52) |
|---|---|---|---|
| Diploid normal | 1.00 ± 0.12 (1,008)* | 1.00 ± 0.11 (4,344) | 1.00 ± 0.10 (1,042) |
| Reg. sq. metapl. | 1.37 ± 0.29 (582) | 1.33 ± 0.32 (954) | 1.40 ± 0.31 (488) |
| Mild atypia | 1.71 ± 0.39 (648) | 1.47 ± 0.25 (1,002) | 1.59 ± 0.45 (552) |
| Moderate atypia | 2.51 ± 0.62 (386) | 1.69 ± 0.70 (655) | 1.94 ± 0.37 (718) |
| Severe atypia | 3.59 ± 1.06 (509) | 2.26 ± 1.05 (315) | 2.62 ± 0.49 (512) |
| Epid. cancer | 5.23 ± 2.44 (912) | 3.57 ± 1.04 (2,251) | 3.41 ± 1.06 (1,277) |

* Parenthetic values = number of cells studied. Significance of interstage QDNA differences within each species = $p < 0.05$.
**Reg. sq. metapl.** = regular squamous metaplasia
**Epid.** = epidermoid

outbred hamsters of mixed/unknown genetic backgrounds. The possibility that various strains of hamsters may have different susceptibilities to topically applied carcinogens had not been explored, and the effects of genetic influences on this method of carcinogenesis had not been assessed. Further, the unpredictable and varying degrees of histocompatibility among outbred hamsters makes them poorly suited for experiments which use serial transplantations of lung cancer allografts. We therefore undertook studies to compare the rates of carcinogenesis between syngeneic strains of hamsters expected to have different susceptibilities to carcinogenesis, and to achieve long-term *in vivo* propagation of lung cancers induced in these strains.

Two varieties of syngeneic hamsters were used for these studies. The LSH strain (Charles River Lakeview Laboratories, Newfield, NJ) is relatively resistant to the oncogenic action of human adenovirus type 12 but had not been used previously in studies of chemical carcinogenesis. However, the LSH strain and the BIO 2.4 strain both originated from the same stock, and the BIO 2.4 strain has been shown to be relatively resistant to gastric and mammary carcinogenesis by MCA administration [14]. The BIO $F_1D$ strain (BIO Breeders Inc., Pittsburgh, PA) is the $F_1$ hybrid from BIO 15.16 males and BIO 87.20 females. The two inbred parental strains are both relatively susceptible to carcinogenesis from polycyclic aromatic hydrocarbons, as is the $F_1D$ [14,15].

SRIs containing 10% B(*a*)P were placed in LSH and $F_1D$ hamsters; SRIs containing 10% MCA were also placed in a group of $F_1D$ hamsters. The hamsters were sacrificed at intervals following SRI placement and the SRI sites examined histologically.

In $F_1D$ animals from both experimental groups, carcinogenesis was observed to proceed at the same rate as that previously observed in outbred hamsters. However, the LSH hamsters were less susceptible to carcinogenesis from 10% B(*a*)P SRIs than were either the $F_1D$ animals or outbred hamsters. This difference appeared to involve a more prolonged period of carcinogen exposure prior to the appearance of squamous metaplasia at the SRI site [16].

Nineteen cancers induced in $F_1D$ hamsters were transplanted into syngeneic hosts; 16 were successfully serially propagated as tumor transplant lines. Although the growth rates of transplants in the recipients varied from one tumor line to another, within each line the growth rate was dependent on the dose of tumor cells transplanted.

To further explore genetic differences and susceptibility to bronchial carcinogenesis and to

## DIFFERENCES BETWEEN HAMSTER STRAINS

| Characteristic | 87.20 | 1.5 | 15.16 |
|---|---|---|---|
| ▪ Coat Color | Tawny | Acromelanic White | Rust |
| ▪ Hepatic N-Acetylation | Fast | Slow | Fast |
| ▪ Sarcoma from Subcut. BP/MCA | ++ | ± | ++ |
| ▪ Gastric CA from MCA Gavage | ++ | ± | ++ |
| ▪ Bladder CA from β-Naphthylamine P.O. | ♂ ± ♀ + | ♂ ± ♀ + | ♂ + ♀ + |

Fig. 2. Known differences of genetic origin among three inbred hamster strains.

## RELATIVE RATES OF SPC AMONG INBRED HAMSTER STRAINS

| Fastest | | Intermediate | | Slowest |
|---|---|---|---|---|
| $F_1D$ ♂ | | $p<0.05$ | | 15.16 ♂ |
| n.s. | | | | |
| $F_1D$ ♀ | | ($F_1D$ = 15.16 ♂ × 87.20 ♀) | | |
| n.s. | | | | |
| 87.20 ♂ | $p<0.02$ | 87.20 ♀ | | |
| n.s. | | | | |
| 1.5 ♂ | | $p<0.05$ | | 1.5 ♀ |

Fig. 3. Rates of SPC among inbred hamster strains and between sexes within strain. Distance of strain/sex designation from left-hand column approximates degree of SPC slowing. Significance of values compares horizontally adjacent varieties; **n.s.** = no significant differences among vertically adjacent varieties.

begin examining the inheritance of relative resistance or susceptibility, B(*a*)P-containing SRIs were placed in both male and female $F_1D$ hybrids, in 15.16 males and 87.20 females (the progenitors of the $F_1D$ hybrid), and in 87.20 males. Also studied were BIO 1.5 males and females; the 1.5 strain had previously been shown to be relatively resistant to chemical carcinogenesis as compared with the other strains [14]. Some known differences among the three inbred strains used are shown in Figure 2.

Groups of hamsters of each variety were sacrificed at 70, 90, 110, 135, 180, 240, and 300 days after SRI placement, and the bronchial epithelium at the SRI site examined histologically. The histologic findings were analyzed using the method of logistic regression analysis [17] developed for use in the studies. The findings are presented in Figure 3. No sex differences were noted in the $F_1D$ strain, but significant sex differences were noted in both the 87.20 and 1.5 strains.

Preliminary genetic analysis of these findings suggests one of the following options: (1) resistance to squamous cell cancer induction from B(*a*)P requires homozygosity for effect, since resistance is different between 15.16 and 87.20 hamsters and absent in the $F_1D$ hybrid from these two strains; or (2) susceptibility to squamous cell carcinogenesis from B(*a*)P is effective in the heterozygote; since susceptibility is different between 15.16 and 87.20 hamsters, it is dominant in the $F_1D$ strain.

Although there were no intra-strain sex differences in the types of cancers induced, the 1.5 strain had a significantly higher incidence ($p < 0.05$) of adenocarcinomas and adenosquamous carcinomas (58%) than did the other varieties of hamsters (21–37%) [18].

## WHAT ABOUT METASTASIS?

The relevance of animal models for human cancer has traditionally been of concern because, although the cancers that developed in the models resemble human cancers histologically, animal cancers did not metastasize as did human cancers. We examined the occurrence of metastasis from the primary tumor in the autochthonous host in our hamster lung cancer model. Autopsies performed on SRI-containing hamsters in the studies described above included a careful search for visible and/or palpable abnormalities of all intrathoracic lymph nodes and all viscera. All such areas were removed and subjected to histologic examination. Because visibly/palpably normal tissues were not routinely examined histologically, the results probably reflect an incidence of metastasis lower than that expected with a more extensive microscopic search. Table IV shows the incidences of metastasis as related to size of the primary lung cancer in 300 hamsters with histologically diagnosed nonsquamous cell lung cancer (NSCLC) [19].

The histological patterns of the metastasizing cancers included adenocarcinoma, adenosquamous carcinoma, squamous cell carcinoma and its spindle cell variant, and large cell carcinoma. Although the numbers are too small for valid correlation between histologic pattern and incidence of metastasis, there was a clear trend for metastasis to occur more often from poorly differentiated cancers. The histologic pattern of all metastases was that of the primary cancer from which they arose.

Reported incidences of metastasis from T1 primary human NSCLC (maximal diameter = 30 mm) vary from 8% to nearly 30% [19]. Because cell sizes of human and hamster tumors are similar, a hamster lesion 10 mm or greater in diameter represents more than 30 doublings of the volume of the original malignant cell, whereas a human lesion of less than 30 mm in diameter (T1) represents less than 35 doublings of the volume of the initial malignant tumor. Therefore the larger hamster NSCLCs metastasize at a similar stage in their development as do the human lesions, *i.e.*, at between 31–34 doublings, or after 31–34 mitotic episodes from the original malignant cell. This similarity between incidence of metastases in humans and in the hamster model provides a further indication that this hamster lung cancer model is relevant for human disease.

As the autochthonous hosts of these tumors were all syngeneic, 26 tumor transplantation lines were developed: six from primary tumors that had metastasized in the autochthonous host, 14 from primary cancers that had not metastasized in the autochthonous host, and six from metastatic lesions [19]. Recipients of these serial dorsal subcutaneous transplants were examined regularly for the presence of metasta-

**TABLE IV. Metastasis From Primary Hamster NSCLCs in Autochthonous Hosts**

| Size of Primary NSCLC | No. Hamsters With Metastasis/ No. Hamsters With Primary NSCLC |
|---|---|
| Microinvasive only | 0/112 |
| Visible <3 mm diam. | 0/66 |
| Visible 3–10 mm diam. | 4/49 (8.2%) |
| Visible >10 mm diam. | 16/73 (22%) |

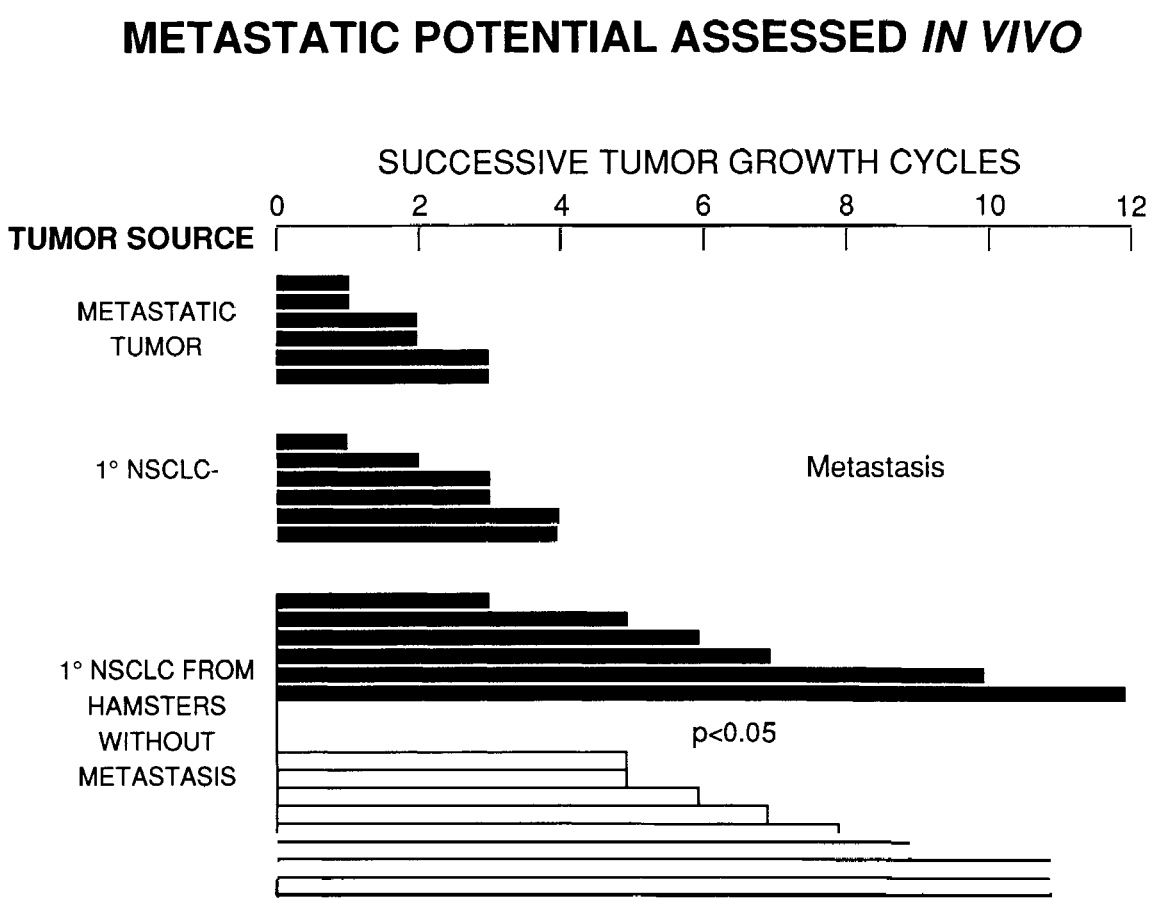


**Fig. 4.** Comparison of numbers of tumor growth cycles until first metastasis detected in transplant recipients as related to metastatic potential of tumor from which transplant line originated. Statistical significance values compare vertically adjacent groups. Each horizontal bar represents a tumor transplant line originating from a different tumor. Solid bars end at the tumor growth cycle during which metastasis occurred; open bars end at the latest tumor growth cycle achieved for that tumor line.

sis. Figure 4 presents the results. If the tumor from which the transplant line originated had already demonstrated the ability to metastasize, metastases occurred early in the transplant recipients. If the tumor from which the transplant line originated had not metastasized in the autochthonous host, six lines took significantly longer to acquire the capability of metastasizing during progressive growth in transplantation, but eight other lines did not metastasize during periods of observation shown in Figure 4. (These eight lines were each observed for 3–5 additional tumor growth cycles; no metastases occurred, and serial transplantation was discontinued.) Even though a heterotopic site of transplantation was used, the correlation between metastases during the serial transplantation and the metastatic proclivity of the cancer used to initiate the tumor transplant line demonstrates the validity of this model for investigations into the biology of the metastatic process.

## INVESTIGATIONS UTILIZING THE HAMSTER LUNG CANCER MODEL

### Non-specific Immunostimulation and Bronchial Carcinogenesis

Earlier investigations to assess the effect of Bacillus Calmette-Guerin (BCG) administration on respiratory carcinogenesis in hamsters were inconclusive [20]. Furthermore, whether or not the BCG regimens used in the studies actually affected immune functions was not determined. In our study, we wished to evaluate the extent of BCG-induced non-specific cellular immunostimulation, and the effect this immunostimulation had on the development of lung cancer in our hamster model. Sixty hamsters were sensitized to keyhole limpet hemocyanin (KLH) as a test substance and half of the hamsters then received BCG intraperitoneally. Finally, all hamsters had a 10% B(*a*)P SRI implanted. The animals were sacrificed in approximately equal numbers at 60, 120, and 180 days following SRI placement. Responses to intradermal test doses of KLH given two days prior to sacrifice were measured, and specimens from the SRI and KLH challenge sites were examined histologically. The BCG-treated group showed significantly greater responses to KLH than did the controls, indicating clear immunopotentiation from BCG. The distribution of preneoplastic/neoplastic lesions at the SRI sites were similar in control and BCG-treated animals at 60 and 120 days after SRI placement, and all animals had NSCLC at the SRI site after 180 days of exposure. All of the cancers in the 180-day control group were visible lesions >1.5 mm diam; however, 75% of the lesions in the BCG-treated, 180-day group were microinvasive cancers, detectable only by microscopic examination ($p < 0.05$). These findings suggest that the documented immunopotentiation from BCG administration had little effect on the rate and incidence of cancer development at the SRI site, but markedly inhibited the subsequent growth of the B(*a*)P-induced cancers [21].

### Carcinogen-altered Bronchi and Adriamycin

One of the enigmas of modern cancer chemotherapy is that tumor cells *in vivo* appear to be relatively resistant to the action of the potent cytotoxins available for clinical use as compared with normal cells. Using the hamster lung cancer model, we examined the interaction between Adriamycin and carcinogen-altered bronchial epithelium.

After varying durations of SRI exposure, which were less than those previously associated with overt cancer, groups of hamsters received intravenous injections of Adriamycin for a 1-minute period. They were then sacrificed at 5, 10, 15, 60, and 360 minutes following the completion of the injection. The SRI site and the corresponding area from the contralateral bronchus were promptly removed, a portion of each was taken for histologic examination, and the remainder was homogenized for Adriamycin determinations. Uptake of Adriamycin into the carcinogen-altered bronchi was no different from uptake into normal bronchi. Although no metabolism of Adriamycin was observed in normal bronchi, 40–60% of the Adriamycin detected in carcinogen-altered bronchi was present as metabolites.

In other experiments, hamsters having undergone similar periods of SRI exposure were sacrificed and the SRI site and contralateral bronchus promptly removed and homogenized. Lipid peroxidation was determined *in vitro*, both in the presence and absence of Adriamycin.

The Adriamycin-induced increase in lipid peroxidation in normal bronchi was nearly four times that found for the carcinogen-altered bronchi [22]. These changes were independent of the duration of exposure to carcinogen from the SRI, the particular carcinogen present in the SRI, and the degree of histological abnormality noted microscopically in the carcinogen-altered bronchi.

These results indicate that some biochemical alterations compatible with a lung cancer's resistance to a chemotherapeutic agent actually originate during the preneoplastic stages of the development of that lung cancer.

## 5-Azacytidine and Hamster Bronchial Carcinogenesis

The cytidine analog, 5-azacytidine (AZC), is a potent inhibitor of the maintenance methylase system in DNA replication. AZC has been widely used in experimental studies of the effects of induced DNA hypomethylation. AZC is also cytotoxic; it is used as a chemotherapeutic agent in some varieties of leukemia, despite evidence that suggests it may itself be mildly carcinogenic. Abnormal levels of methylated cytosine, usually hypomethylation, have been found in a wide variety of cancers. Using our hamster model, we examined the effect of systemic AZC administration on B(*a*)P-induced chemical carcinogenesis; we also explored the carcinogenicity of AZC itself in hamsters.

In the first study, outbred hamsters received a SRI containing 10% B(*a*)P; half received no further treatment and half received AZC injections (5 mg/kg, ip) twice weekly throughout the remainder of the experiment. Cohorts from both groups were sacrificed at 3-week intervals beginning 61 days following SRI placement. The bronchial epithelium at the SRI site was examined histologically, as was the contralateral bronchial epithelium in the AZC-treated hamsters. The previously defined, and reproducible, SPC was found in both the controls and the AZC-treated hamsters. However, when compared by logistic regression analysis, the SPC was slower in AZC-treated hamsters, and the size of the cancers that developed was significantly less than in the controls. Because of concern regarding the possible effect of varying susceptibility to carcinogenesis in outbred hamsters, and because we suspected that the findings might be due to a chemotherapeutic-like action of AZC on very early cancers, we conducted a second study.

In the second study, inbred (syngeneic) hamsters were used, eliminating differential susceptibility to carcinogenesis. All hamsters received a 10% B(*a*)P SRI. One group had no further treatment; a second group received AZC twice weekly throughout the experiment, as in the first study. A third group received AZC twice weekly for only the 80 days following the SRI placement; the fourth group received no AZC until 80 days following SRI replacement, at which time twice-weekly injections of the same dose of AZC were begun and continued throughout the rest of the experiment. From each of the four groups, cohorts were sacrificed at 80, 150, 180, and 220 days after SRI placement. The bronchial epithelium at the SRI site and at similar areas in the contralateral bronchus was examined histologically.

The following parameters were determined for each group of hamsters: the rate of the SPC, the eventual incidence of cancers, the distribution of epidermoid and nonepidermoid cancers, the sizes of the cancers, and the degree of differentiation within the histologic pattern of the cancers. With respect to these parameters, there was no difference between the controls that received only the B(*a*)P SRI and the hamsters that also received AZC late, *i.e.*, beginning at 80 days after SRI placement. Also, there was no difference in these parameters between the hamsters that received AZC continuously and those that only received it for the first 80 days. However, in the continuous and early AZC treatment groups, most parameters were significantly different from the control and late AZC groups. The rate of the SPC was slower, the cancers were smaller and better differentiated, and there were fewer nonepidermoid cancers. These results dispelled our concern about the findings of the first study suggesting a chemotherapeutic-like effect of AZC on early cancers. Furthermore, it was clear that the effects of AZC treatment on the cancers was a consequence of its administration during the preneoplastic stages, because only those groups of hamsters that received AZC during that period showed the later changes [23].

We also examined the total cellular DNA content (QDNA) of cells at each of the preneoplastic stages of the SPC and compared the values between AZC-treated animals and controls. During the first part of the SPC, including moderate atypical squamous metaplasia, QDNA values were significantly greater in the AZC-treated animals than in the controls, but the QDNA values for severe atypical metaplasia and for cancer were less in the AZC-treated animals than in the controls. This finding was the same for both varieties of hamster, outbred and syngeneic [24].

Although the mechanisms of interactions between B(*a*)P and AZC during the preneoplastic stages of SPC remain to be determined, it is clear that significant changes in the rate of the SPC, QDNA values of resulting cancers, and their histologic patterns and degrees of differentiation are all consequences of the B(*a*)P-AZC interaction during preneoplastic stages of the SPC, manifested by the difference in QDNA values of the early SPC stages as compared with later SPC stages. This finding is similar to that described above for the Adriamycin studies, in that characteristics of eventual cancer are determined by changes transpiring during the preneoplastic stages of that cancer. Whether this phenomenon exists for all the various differences present between normal tissues and malignancies thereof remains to be seen.

With respect to the tumorigenicity of AZC, 194 hamsters received it systemically for periods up to 70 weeks. Among these hamsters, only one tumor was detected at sites other than that of the SRI placement; this was a malignant histiocytoma of the thigh. In these same groups of animals, there were 248 lungs without B(*a*)P-SRI; no gross or microscopic cancer, atypia, or squamous metaplasia was detected. At the dose used, AZC (5 mg/kg ip, biweekly) was clearly noncarcinogenic.

## REGRESSION OF BRONCHIAL CANCER

For bronchogenic carcinoma, the questions of *if* and *when* the SPC is reversible are fundamental to chemoprevention research. A recent addition to our hamster lung cancer model is the development of a method for removing the endobronchial SRI without loss of the hamster. In this study, 114 hamsters received removable B(*a*)P-SRIs. Short-term controls were sacrificed at 50, 65, and 80 days after the SRI placement. Additional experimental groups had SRIs removed at 50, 65, and 80 days after placement and sacrifice was delayed until 100–180 days later. Long-term controls retained the SRIs until sacrifice at 180 or 240 days after SRI placement. SRI sites were examined histologically; the findings are shown in Figure 5.

All long-term controls had NSCLC. Preneoplastic change was more common in 50 and 65 day controls, as compared with hamsters with equal duration of SRI exposure whose sacrifice was delayed until 100–180 days after SRI removal ($p < 0.05$). The 56% incidence of early NSCLC in hamsters sacrificed after 80 days of SRI exposure decreased to 5% in hamsters that had delayed sacrifice following SRI removal after 80 days of exposure ($p < 0.05$). At the B(*a*)P dose used, hamster bronchial epithelium requires >80 days of continuous exposure uniformly to become irreversibly committed to NSCLC. Microinvasive NSCLC in hamsters may regress; it does not necessarily progress to overt invasive cancer. The removable SRI model provides new opportunities to evaluate chemoprevention of NSCLC and the related molecular genetic control mechanisms [25].

## SUMMARY

We believe the studies described above show that our hamster lung cancer model meets the criteria set forth at the beginning of this report. Variables that may be readily examined under controlled conditions with this model include: (1) the particular carcinogen and dose; (2) the duration of carcinogen exposure; (3) the susceptibility of the carcinogen-exposed animal; and (4) the actions of dietary alterations or of exogenously administered substances, either of which may affect carcinogenesis.

In the model, we have primarily used B(*a*)P, the polycyclic aromatic hydrocarbon carcinogen most likely involved in causing human lung cancer. Relevance of our model to human lung cancer is also manifested in the following ways:

1. The exposure to carcinogen is endobronchial, as with an inhaled agent.
2. The pathogenesis of epidermoid cancer in the model is like that seen in hu-

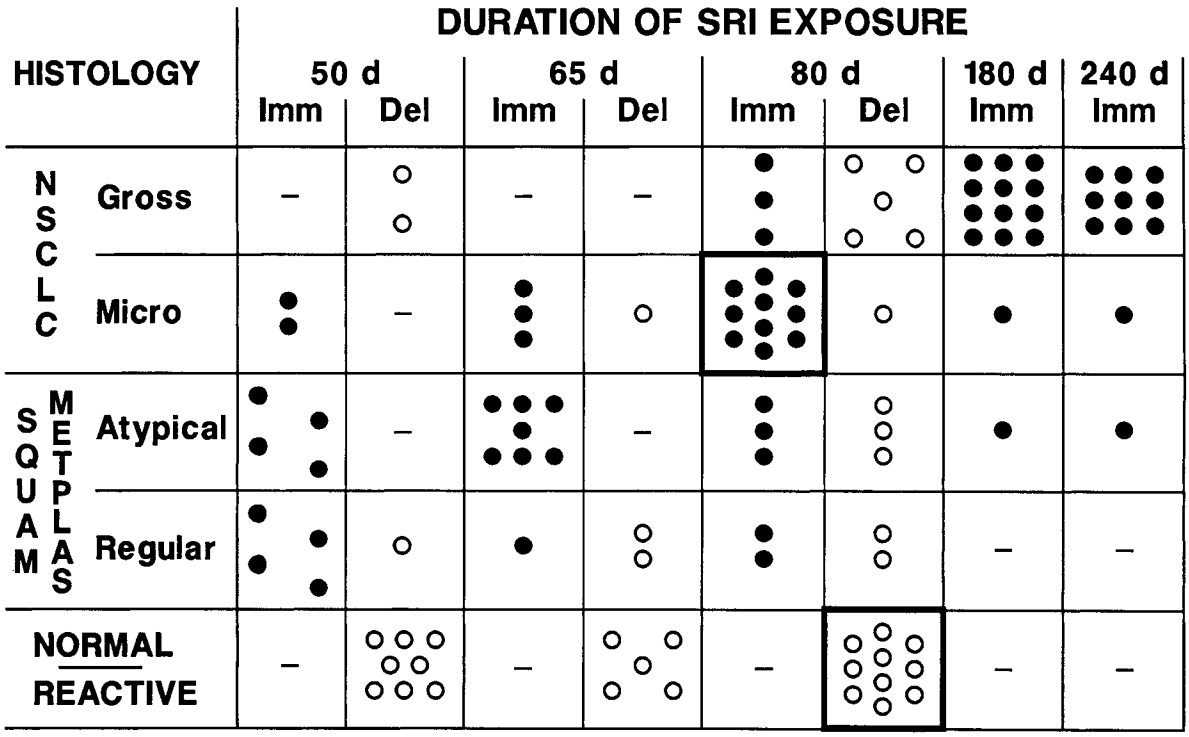


**Fig. 5.** Each dot represents the most severe histological abnormality found at the SRI site in a single hamster. Solid dots represent hamsters sacrificed immediately (imm) after the indicated SRI exposure duration; open dots represent those hamsters in which sacrifice was delayed (del) for 100–180 days after SRI removal terminated the indicated exposure duration. Histology reflects progressive stages of SPC as defined in text. Bold lines highlight most significant difference between immediate and delayed sacrifice groups.

mans, including the progressively increasing ploidy typical of the SPC.

3. Cancers in the model are histologically similar to those seen in humans and show the same ploidy abnormalities. (One of the enigmas of respiratory carcinogenesis is that small cell lung cancer has not yet been reproducibly induced in any animal model.)
4. Metastases from the primary cancer occur via a similar path, during a similar number of tumor cell doublings, and with a similar incidence as those in humans.
5. Varying susceptibility to the same carcinogenic stimulus exists in the model among inbred strains, similar to the variable susceptibility which occurs in humans.

Hence, the hamster model is a uniquely useful tool for seeking an increased understanding of processes involved in the progression of non-small cell bronchogenic cancer, from the earliest change in the respiratory epithelium to a lethal outcome from metastatic disease.

## REFERENCES

1. Roe FJ: The relevance and value of studies of lung tumors in laboratory animals in research on cancer of the human lung. In Severi L (ed): "Lung Tumors in Animals." Perugia, Italy: University of Perugia, 1966, pp 101–126.
2. Shors E, Fu PC, Matsumura K, Cohen AH, Benfield JR: Sustained release of benzo(*a*)pyrene from silicone polymer into the tracheobronchial tree of hamsters and dogs. Cancer Res 40:2288–2294, 1980.
3. Saffiotti U, Cefis F, Kolb LH: A model for the experimental induction of bronchogenic carcinoma.

Cancer Res 28:104–124, 1968.
4. Saccomanno G, Archer VE, Auerbach O, Saunders RP, Brennan LM: Development of carcinoma of the lung as reflected in exfoliated cells. Cancer 33:256–270, 1974.
5. Benfield JR, Shors E, Hammond WG, Paladugu RR, Cohen AH, Jensen T, Fu PC, Pak HY, Teplitz RL: A clinically relevant canine lung cancer model. Ann Thorac Surg 32:592–601, 1981.
6. Stenback F: Morphology of experimentally induced respiratory tumors. Acta Otolaryngol 347(Suppl): 1–59, 1977.
7. Benfield JR, Hammond WG: Focal bronchial and pulmonary carcinogenesis in dogs and hamsters. Cancer Res 52:(Suppl)2687s–2693s, 1992.
8. Benfield JR, Hammond WG, Shors EC, Paladugu RR, Pak HY, Teplitz RL: A family of epidermoid lung cancer models. Ann Thorac Surg 38:627–632, 1984.
9. Nettesheim P, Klein-Szanto AJP, Marchok AC, Steele VE, Terzaghi M, Topping DC: Studies of neoplastic development in respiratory epithelium. Arch Pathol Lab Med 105:1–10, 1981.
10. Peck RM, Tan TK, Peck EB: Pulmonary carcinogenesis by derivatives of polynuclear aromatic alkylating agents. Cancer Res 36:2423–2427, 1976.
11. Gross P, Tolker E, Babyak MA, Kaschak M: Experimental lung cancer in hamsters. Arch Environ Health 11:59–65, 1965.
12. Hammond WG, Shors E, Paladugu RR, Cohen A, Fu P, Benfield JR: Intercarcinogen comparison in hamster lung cancer. Proc Am Assoc Cancer Res 23:56, 1982.
13. Pak NY, Teplitz RL, Ashdjian V, Yokota SB, Hammond WG, Benfield JR: Quantitative DNA determination by image analysis. II. Application to human and canine pulmonary cytology. Anal Quant Cytol Histol 5:263–268, 1983.
14. Homburger F, Hsueh S-S, Kerr CS, Russfield AB: Inherited susceptibility of inbred strains of Syrian hamsters to induction of subcutaneous sarcomas and mammary and gastrointestinal carcinomas by subcutaneous and gastric administration of polynuclear hydrocarbons. Cancer Res 32:360–366, 1972.
15. Homburger R, Adams RA, Bernfeld P, Van Dongen CG, Soto E: A new first-generation hybrid Syrian hamster, B10, $F_1D$ Alexander, for *in vivo* carcinogenesis bioassay, as a third species or to replace the mouse. Surv Synth Pathol Res I:125–133, 1983.
16. Hammond WG, Gabriel A, Paladugu RR, Azumi N, Hill R, Benfield JR: Differential susceptibility to bronchial carcinogenesis in syngeneic hamsters. Cancer Res 47:5202–5206, 1987.
17. Hill LR, Hammond WG, Benfield JR: Logistic regression analysis in experimental bronchial carcinogenesis. Am Stat 45:184–186, 1991.
18. Hammond WG, Cohen AH, Teplitz RL, Benfield JR: Adenocarcinoma among syngeneic hamsters with differential susceptibility to non-small cell lung cancer (NSCLC). Proc Am Assoc Cancer Res 31:84, 1990.
19. Hammond WG, Teplitz RL, Benfield JR: Lung cancer model for study of the metastatic process. Ann Thorac Surg 52:732–737, 1991.
20. Zwilling BS, Davis GW: Effect of Bacillus Calmette-Guerin on the development of primary lung cancer in Syrian golden hamsters. Cancer Res 40:3455–3458, 1980.
21. DeCaro LW, Paladugu RR, Benfield JR: Influence of BCG on lung carcinogenesis. Proc Am Assoc Cancer Res 23:243, 1982.
22. Carr BL, Langley D, Dias CB, Hammond WG, Benfield JR: Differential interaction of normal and preneoplastic hamster bronchi with Adriamycin. Cancer Res 46:2730–2734, 1986.
23. Hammond WG, Yellin A, Gabriel A, Paladugu RR, Azumi N, Hill LR, Benfield JR: Effects of 5-azacytidine in Syrian Golden hamsters: Toxicity, tumorigenicity, and differential modulation of bronchial carcinogenesis. Exp Mol Pathol 53:34–51, 1990.
24. Hammond WG, Yellin A, Gabriel A, Azumi N, Hill LR, Teplitz RL, Benfield JR: Quantitative DNA alterations during 5-azacytidine-induced differential modulation of benzo(*a*)pyrene carcinogenesis in hamster bronchi. Cancer Commun 2:135–144, 1990.
25. Sawyer RW, Hammond WG, Teplitz RL, Benfield JR: Regression of bronchial epithelial cancer in hamsters. Ann Thorac Surg, 1993 (in press).