A PRACTICAL MODEL OF BRONCHOGENIC
CARCINOMA IN CAMM-HARTLEY GUINEA
PIGS AND GOLDEN SYRIAN HAMSTERS

By

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We accept this thesis as conforming
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ABSTRACT

Bronchogenic carcinoma (lung cancer) is a major source of morbidity and mortality in industrialized nations. Although bronchogenic carcinoma is largely a preventable neoplasm, it will undoubtedly remain a major medical concern throughout this century, and considerable effort needs to be directed towards its early detection, establishing effective treatment, and understanding the neoplastic process.

The objective of this study was to develop a practical and reliable model of localized bronchogenic carcinoma in laboratory rodents. This was done by impregnating cotton threads with the potent carcinogen, benzo(a)pyrene (BP). These BP-impregnated threads were then coated with a silicone rubber sheath to control the release of BP from the threads. The prepared threads were sewn around four cartilage rings in the ventral tracheal wall of guinea pigs and hamsters. The animals were sacrificed periodically, and two histopathologists graded the tracheal epithelium adjacent to the thread.

The first experiment consisted of 94 Camm-Hartley guinea pigs: 48 experimental animals with BP-impregnated threads and 46 control animals with non-impregnated threads. The second experiment consisted of 70 Golden Syrian hamsters: 54 experimental and 16 control animals. The data showed that the implantation of the thread in the trachea induced a regenerative hyperplasia of the epithelium, and that the BP initiated carcinogenesis. Squamous metaplasia and progressive intraepithelial neoplasia (IEN) was evident prior to the development of squamous cell carcinoma (CA). In the experimental guinea pigs, only one guinea pig developed an invasive CA at 265 days. In the
experimental hamsters, the first CA was seen at 55 days and after 120 days, 65% of the animals showed histopathologic evidence of CA. Most of the hamsters with CA also had spindle cell tumors in the tracheal stroma. The control hamsters and guinea pigs did not develop IEN, and the mature respiratory epithelium was reconstituted.

We conclude that this method produced localized, readily accessible preneoplastic and neoplastic lesion in the trachea of hamsters, and to a lesser extent in guinea pigs. The model should prove useful in the study of tumor ultrastructure, the immunologic response to cancer, and the relationship of diet to cancer.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>x</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xi</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>xii</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>A. Bronchogenic Carcinoma</td>
<td>1</td>
</tr>
<tr>
<td>B. Bronchogenic Carcinoma and Cigarette Smoking</td>
<td>2</td>
</tr>
<tr>
<td>C. Cigarette Smoke</td>
<td>4</td>
</tr>
<tr>
<td>D. Multistage Theory of Carcinogenesis</td>
<td>5</td>
</tr>
<tr>
<td>E. Benzo(a)pyrene Metabolism</td>
<td>6</td>
</tr>
<tr>
<td>F. Animal Models of Bronchogenic Carcinoma</td>
<td>10</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>14</td>
</tr>
<tr>
<td>A. Animals</td>
<td>14</td>
</tr>
<tr>
<td>B. Threads</td>
<td>14</td>
</tr>
<tr>
<td>C. Surgical Procedures</td>
<td>15</td>
</tr>
<tr>
<td>D. Experiments</td>
<td>16</td>
</tr>
<tr>
<td>E. Preparation of the Tracheas</td>
<td>16</td>
</tr>
<tr>
<td>F. Evaluation of the Tracheas</td>
<td>17</td>
</tr>
</tbody>
</table>
G. Livers, Lungs and Lymph Nodes........................................ 18
H. Animal Survival....................................................... 19

RESULTS............................................................................. 20
A. Benzo(a)pyrene Content of the Threads.............................. 20
B. Histopathology of the Tracheas......................................... 20
C. Histopathology of the Tracheal Epithelium in the Control
   Animals........................................................................... 21
D. Histopathology of the Tracheal Epithelium in the
   Experimental Animals................................................... 21
E. Spindle Cell Tumors....................................................... 24
F. Evaluation of the Histopathology of the Tracheal Epithelium... 25
G. Efficacy of Different Thread Options to Induce Squamous
   Cell Carcinoma............................................................. 27
H. Inflammation and Keratinization of the Tracheal Epithelium... 27
I. Complications of the Model.............................................. 28
J. Lymph nodes, Liver and Lung......................................... 28
K. Survival Data.............................................................. 30

FIGURES AND TABLES.......................................................... 31

DISCUSSION..................................................................... 49
A. Consideration of the Histopathologic Results...................... 49
B. Efficacy of Different Thread Options to Induce Squamous
   Cell Carcinoma............................................................. 52
C. Tissue Origin of the Spindle Cell Tumor............................. 54
D. The Role of Inflammation, Regenerative Hyperplasia and
   Keratinization of the Tracheal Epithelium in Tumor Promotion.. 57
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Assessment of the Overall Interobserver Error in the Histopathologic Grading</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>of the Tracheal Epithelium</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Efficacy of the Different Thread Options to Induce Squamous Cell Carcinoma</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>in Hamsters</td>
<td></td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Impregnation of Cotton Threads with Benzo(a)pyrene</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>Preparation of the Silicone Rubber Sheath</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>Apparatus used to Coat the Threads with a Silicone Rubber Sheath</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>Diagram of the Surgical Procedure Performed on the Rodents</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>Diagram of a Tumor Forming Around the Thread within the Ventral Tracheal Wall</td>
<td>33</td>
</tr>
<tr>
<td>6</td>
<td>Low Power Photomicrograph of a Thread within the Tracheal Wall</td>
<td>33</td>
</tr>
<tr>
<td>7 &amp; 8</td>
<td>Low and High Power Photomicrographs of the Proliferating Epithelium in a Guinea Pig Control, 4 Days Post-Operation</td>
<td>35</td>
</tr>
<tr>
<td>9</td>
<td>Regenerating Squamous Epithelium in a Guinea Pig Control, 20 Days Post-Operation</td>
<td>35</td>
</tr>
<tr>
<td>10 &amp; 11</td>
<td>Low and High Power Photomicrographs of Mature Tracheal Epithelium in a Guinea Pig Control, 183 Days Post-Operation</td>
<td>35</td>
</tr>
<tr>
<td>12</td>
<td>Normal Guinea Pig Tracheal Epithelium</td>
<td>37</td>
</tr>
<tr>
<td>13</td>
<td>Squamous Metaplasia and Keratinization in an Experimental Hamster</td>
<td>37</td>
</tr>
<tr>
<td>14</td>
<td>Mild Intraepithelial Neoplasia in an Experimental Hamster</td>
<td>37</td>
</tr>
<tr>
<td>15</td>
<td>Moderate Intraepithelial Neoplasia in an Experimental Hamster</td>
<td>37</td>
</tr>
<tr>
<td>16 &amp; 17</td>
<td>Marked Intraepithelial Neoplasia in an Experimental Hamster</td>
<td>37/38</td>
</tr>
</tbody>
</table>
18 & 19. Low and High Power Photomicrographs of a Squamous Cell Carcinoma in a Guinea Pig Trachea................................. 40
20. Invasive Squamous Cell Carcinoma in a Hamster Trachea........ 40
21. Squamous Cell Carcinoma Merging with a Spindle Cell Tumor in a Hamster Trachea.................................................. 40
22. High Power Photomicrograph of a Spindle Cell Tumor in a Hamster Trachea................................................................. 40
23. Gross Appearance of a Tracheal Tumor in a Hamster.............. 40
24. Histopathologic Data of Guinea Pig Tracheal Epithelium........ 43
25. Histopathologic Data of Hamster Tracheal Epithelium............ 43
26. Bar Graph of Guinea Pig Survival...................................... 47
27. Bar Graph of Hamster Survival........................................ 47
### LIST OF ABBREVIATIONS

- **AHH** - aryl hydrocarbon hydroxylase
- **BP** - benzo(a)pyrene
- **C** - cartilage
- **CA** - squamous cell carcinoma
- **Con** - control animals (not exposed to BP)
- **DTW** - dorsal tracheal wall
- **Exp** - experimental animals (exposed to BP)
- **F** - fibrosis
- **IEK** - intraepithelial keratin
- **IEN** - intraepithelial neoplasia (1-mild, 2-moderate & 3-marked)
- **K** - keratin (superficial)
- **LM** - light microscopy
- **ME** - mature epithelium
- **PAH** - polycyclic aromatic hydrocarbons
- **PMN** - polymorphonuclear leukocytes
- **S** - stroma (submucosa, cartilaginous layer & adventitia of trachea)
- **SCT** - spindle cell tumor
- **SM** - squamous metaplasia
- **SRS** - silicone rubber sheath
- **TD** - thread
- **TEM** - transmission electron microscopy
- **TL** - tracheal lumen
- **TU** - tracheal tumor
- **VTW** - ventral tracheal wall
I would like to thank my supervisor, Dr. J.C. Hogg, for providing the opportunity to work in his laboratory, for offering his time and constructive criticisms, and for his idea of inserting carcinogen-impregnated threads into rodent tracheas to produce tumors.

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INTRODUCTION

Bronchogenic Carcinoma

Bronchogenic carcinoma (lung cancer) is a major source of morbidity and mortality in industrialized nations. Presently, it is the most common cause of cancer-related death in males and within the next decade, it is projected to exceed breast carcinoma as the most common cause of cancer-related death in women; the overall five year survival rate of bronchogenic carcinoma is less than 10% (Silverberg, 1983). The major problem is that the localized bronchial lesions metastasize early to the regional lymph nodes and other organs of the body. Due to the rapid progression of the disease, metastatic carcinoma is seen in 50% of patients at the time of the initial presentation (Van Houtte et al., 1983). This tumor can be potentially cured by surgery, yet the overall rate of cure has improved little since the introduction of surgical treatment (Wilkins et al., 1978). Radiotherapy and chemotherapy may prolong survival in some patients, but are not curative (Van Houtte et al., 1983). The fact that this cancer passes quickly through the stage where it is localized and operable to the stage where it is invasive and inoperable has frustrated attempts at early detection and treatment.

Since most lung cancers are bronchogenic carcinomas, (accounting for 90-95% of lung cancers), the two terms are often used interchangeably. The remaining 5 to 10% of lung cancers are bronchial carcinoids (or bronchial adenomas), mesenchymal and other miscellaneous neoplasms. Bronchogenic carcinomas are classified into the following histologic types: squamous cell (epidermoid) carcinoma (variant-spindle cell carcinoma), adenocarcinoma (acinar and papillary carcinomas, and bronchioloalveolar carcinoma), small
cell carcinomas (oat cell and spindle or polygonal small cell), large cell carcinomas (variants—giant cell and clear cell carcinoma) and adenosquamous carcinoma (combined squamous cell carcinoma and adenocarcinoma). Not all bronchogenic carcinomas are of bronchial origin, as the term suggests. For example, adenocarcinomas are usually peripherally located (bronchiolar or bronchioloalveolar origin), as opposed to the more central hilar (bronchial) location of most squamous cell and small cell carcinomas. (More detailed information about human bronchogenic carcinomas is available in Shimosato et al., 1981, and Carter et al., 1980).

In the model of bronchogenic carcinoma reported here, the trachea serves as the carcinogenic substrate. In humans, malignant tracheal tumors are comparatively rare, most represent direct extension from bronchial or esophageal carcinoma. Yet primary tracheal tumors are reported, usually squamous cell or adenoid cystic carcinomas (Houston, 1969). The tracheal epithelium is in continuity with the bronchial epithelium and has a similar array of cells: basal cells to regenerate the epithelium, ciliated columnar cells to sweep the intrapulmonary debris up and out, and goblet cells to provide the mucous layer which coats the tracheobronchial tree. Although the anatomical location of bronchus and trachea differ, the epithelium is similar, and the cellular response to carcinogens is similar in human bronchus and hamster trachea (Autrup et al., 1980).

Bronchogenic Carcinoma and Cigarette Smoking

It is well recognized that a causal relationship exists between chronic cigarette smoking and bronchogenic carcinoma. This fact is based on prospective and retrospective epidemiological studies, clinical studies,
autopsy series and experimental studies in animals (United States Department of Health, 1979).

Epidemiological studies show that the risk of developing bronchogenic carcinoma is, on the average, 10 times greater for smokers than for non-smokers (Hammond and Horn, 1958). This risk increases with heavy cigarette consumption, and with occupational exposure to chromates, nickel, asbestos and uranium (Frank, 1982). The causal relationship between smoking and bronchogenic carcinoma has been strengthened by delineating dose-response relationships: the risk for bronchogenic carcinoma increases with the number of cigarettes per day, and the duration of smoking (Hammond and Horn, 1958). The risk of developing lung cancer does decline for ex-cigarette smokers compared to continuing cigarette smokers (Wynder, 1972).

The clinical evidence suggesting that cigarette smoking causes lung cancer centers around studies of the respiratory epithelium in smokers compared to non-smokers. The respiratory epithelium of chronic cigarette smokers is believed to proceed through a progressive series of preneoplastic changes before the development of bronchogenic carcinoma (Auerback et al., 1962). These preneoplastic changes (epithelial dysplasia or intraepithelial neoplasia) have been extensively studied in human cervical epithelium (Richart et al., 1982 & Buckley et al., 1982). Clinical studies show that the early antecedants of bronchogenic carcinoma, epithelial hyperplasia and dysplasia, are seen in a greater proportion of smokers than in comparable non-smokers (Auerbach et al., 1979).

Experimental animal models of tobacco smoke induced carcinogenesis constitute a crucial link in the chain of evidence supporting the causal relationship of cigarette smoking to bronchogenic carcinoma. The earliest
animal models involved repeated skin painting of tobacco tar and some of the
condensate subfractions of tobacco smoke (Orris et al., 1958, and Murphy and
Sturm, 1925). Then various fractions of cigarette smoke condensate were
inoculated into the lungs of rats, with only moderate success (Blacklock and
Burgan, 1962, and Blacklock, 1961). Later, more sophisticated models of
cigarette smoke induced lung cancer were conducted in dogs (Auerback et al.,
1970). In general, although bronchogenic carcinoma can be induced in animal
models with cigarette smoke or tobacco tar condensates, the yields are low
(Kuschner and Laskin, 1971).

Cigarette Smoke

The combustion of tobacco generates several hundred compounds by a
variety of chemical and physical processes (Wynder and Hoffmann, 1967). To
aid the analysis of tobacco smoke, its constituents have been separated into a
gas phase (defined as those substances which will pass through a Cambridge
filter pad which is 99.9% efficient for particles more than 0.1 microns in
diameter) and a particulate phase (defined as those substances which collect
on the filter). The gas phase contains carbon monoxide, carbon dioxide,
nitrogen oxides, ammonia, volatile N-nitrosamines and hydrogen cyanide. The
particulate phase consists of water, nicotine and tar. The tar is primarily
composed of a myriad of polycyclic aromatic hydrocarbons (PAH), to which
carcinogenicity is attributed. Some of the PAH include benzopyrenes,
chrysenes, and anthracenes. The tar also contains non-volatile N-nitroamines,
aromatic amines, isoprenoids, benzenes, naphthalenes, aza-arenes, phenols,
carboxylic acids, metallic ions, and radioactive compounds (potassium-40,
lead-210, polonium-210 and radium-226).
The PAH are only a small fraction of a very complex mixture. Nevertheless, fractionation studies with tobacco tar have shown that those fractions enriched in PAH induce tumors in animals (Orris et al., 1958, and Blacklock and Burgan, 1962). Over 100 individual PAH have been identified, but the classic PAH, benzo(a)pyrene (BP) and dibenz(a,h)anthracene, stand out as being major carcinogens in cigarette smoke. Yet other constituents contribute to the carcinogenic potential of cigarette smoke. For example, non-volatile N-nitroamines are formed during curing, fermentation and combustion of tobacco; these chemicals are known carcinogens (Hecht et al., 1978, and Hilfrich et al., 1977). It must be appreciated that the amount of these carcinogens in cigarette smoke is very low. There is about 25 ng of BP per cigarette smoked (Wynder and Hoffman, 1967), necessitating repeated exposures for many years before bronchogenic carcinoma develops.

Multistage Theory of Carcinogenesis

When the chemical components of cigarette smoke are tested in isolation, many are not active as complete carcinogens (able to induce cancers by themselves). Rather, certain chemical agents play interactive roles in carcinogenesis, and often a set of chemicals is required to produce experimental cancers. To explain this phenomenon, the multistage theory of carcinogenesis was devised, in which at least two distinct independent steps are necessary to transform a normal cell to a malignant cell (Pitot, 1982 and Farber, 1981). A tumor initiator is an agent which induces a dormant tumor cell, likely by a direct attack of the initiating agent on the DNA of the cell. Initiation is generally considered to be completed relatively rapidly and to be essentially irreversible. A tumor promoter is an agent which allows
expression of the dormant tumor cell by selective proliferation of the initiated cells. Tumor promoters are effective only if applied to the tissue after a prior treatment with the tumor initiator. In contrast to initiating agents, promoting agents exhibit a reversibility in their action, and this may be modulated by diet, hormonal and other environmental factors. This has important implications for human cancers, as tumor promotion can potentially be manipulated after preventive strategies to avoid tumor initiation have failed.

A co-carcinogen is a chemical which is neither a tumor initiator, a tumor promoter, nor a complete carcinogen, but it is capable of augmenting the carcinogenic response of tissue to a minimal dose of carcinogen. A later stage in tumor development in which the tumor mass grows, begins to invade, and potentially metastasizes is often delineated, referred to as tumor progression.

The various chemicals in cigarette smoke probably operate in concert to produce carcinomas, each operating at different stages in carcinogenesis. BP's tumor initiating potential is enhanced by promoting agents. Other PAH, such as pyrene and benzo(g,h,i)fluoranthene, are thought to be co-carcinogens; whereas the weakly acidic fraction of cigarette smoke contains phenols which are tumor promoters, akin to the tumor promoter prototype, phorbol ester, in mouse skin (Slaga and Klein-Szanto, 1983 and Slaga et al., 1982).

Benzo(a)pyrene Metabolism

Benzo(a)pyrene (BP) is not a carcinogen in its native form, it must undergo metabolism in the microsomal (smooth endoplasmic reticulum) monooxygenase (mixed function oxidase or cytochrome P-450) enzyme system. This enzyme system is usually responsible for detoxification of foreign
chemicals, but with some chemical carcinogens, this same system creates active chemical carcinogens (Miller, 1978). Two metabolic routes of carcinogenicity are possible with BP. The most intensively investigated is the direct mechanism of BP carcinogenesis in which the proximate carcinogen (native BP) is metabolized by monooxygenase enzymes to a simple epoxide; this epoxide is hydrolyzed to a dihydrodiol by epoxide hydrolase (epoxide hydratase), and finally the ultimate carcinogen is formed, a diol-epoxide derivative of BP, by another activation step by monooxygenase enzymes (Conney, 1982, Sims, 1980 and Heidelberger, 1977). Several different diol-epoxides are formed, but it is believed that the (+)-anti-BP-7,8-diol-9,10 epoxide is the most biologically active (Slaga et al., 1979). This electrophilic reactant then exerts its carcinogenic effect by covalent interaction with cellular macromolecules, namely by forming an adduct with guanine in DNA (Phillips, 1983). How this in turn results in a malignant cellular phenotype is not understood.

The other metabolic route which BP may follow is the indirect mechanism of carcinogenicity in which free radical intermediates are generated when BP is shuttled through the monooxygenase enzyme system (Menger et al., 1976, Wilk and Girke, 1972, and Tso et al, 1977). These BP free radical intermediates engender oxygen-free radicals which can then damage cellular macromolecules and lead to carcinogenesis (Cavalieri et al., 1976, Ide et al., 1983 and Cerutti and Remsen, 1977).

The relative role of the direct and indirect mechanism of BP carcinogenicity remains to be elucidated. Furthermore, each of these two metabolic pathways generate a spectrum of products, which may have additive, synergistic and perhaps even opposing actions.

BP is known to be a complete carcinogen; that is, it is inherently
able to produce both tumor initiation and promotion. The activation of BP leading to DNA adduct formation (direct mechanism), if coupled with one cycle of cell proliferation (a property tumor promoters), will result in tumor initiation (Farber, 1981). On the other hand, oxygen free radicals (formed in the indirect mechanism) are believed to result in tumor promotion (Cerutti, 1985 and Goldstein et al., 1983). Perhaps the two different mechanisms of BP carcinogenicity play separate roles in the multistage induction of cancer, but this may be overly simplistic and has yet to be proven.

The metabolism of BP in human lungs has received much attention in the past. This is because BP has been implicated as one of the major carcinogens in cigarette smoke causing bronchogenic carcinoma. Although this relationship is generally accepted, one of the enigmas about cigarette smoking and lung cancer is that only a sub-fraction of smokers develop lung cancer. The reason for this is not known, but in the early 1970's an appealing theory was proposed, which stated that susceptibility to bronchogenic carcinoma is associated with higher levels of inducible aryl hydrocarbon hydroxylase (AHH) activity. The polycyclic aromatic hydrocarbons (PAH) in general, and BP in particular, are metabolized in human cells by a complex of microsomal enzymes (monooxygenase and epoxide hydrolase or AHH) to products capable of inducing neoplastic transformation (Milo et al., 1978). AHH activity is known to be induced by cigarette smoke and by purified PAH (Welch et al., 1971). Further, it has been shown that variation in the extent of AHH induction is under genetic control; AHH inducibility is determined by a single gene with additive expression of two alleles (Kellermann et al., 1973a). Kellermann reported (Kellermann et al., 1973b) that patients with bronchogenic carcinoma show a selection in gene frequencies which allows higher levels of inducible AHH
activity when compared to controls. Subsequent studies refuted this evidence (Paigen et al., 1977). The prototype tissue for determination of AHH activity in man has been cultured peripheral blood lymphocytes. The inconsistencies in the reported human AHH activity in patients with bronchogenic carcinoma might be due to sampling errors, day to day variation in lymphocyte AHH activity, different lymphocyte culture conditions and different AHH assays. In an effort to provide a more reproducible measure of AHH activity, human pulmonary alveolar macrophages from lavage in combination with cultured human lymphocytes have been assayed, and these studies also showed that lung cancer patients have higher levels of inducible AHH activity than patients without lung cancer but with comparable smoking histories (McLemore et al., 1981). Because these studies support an attractive theory, namely that susceptibility to bronchogenic carcinoma is inherited as an augmented ability to metabolize chemical carcinogens, it is tempting to accept the aforementioned evidence. But peripheral blood lymphocytes and alveolar macrophages are an indirect means of assessing the capacity of the bronchial epithelium to activate chemical carcinogens. Prospective investigation of the AHH activity in human bronchus, in properly controlled patient groups, would be required before this theory gains general acceptance.

Given that carcinogens such as BP must be metabolized by the mono-oxygenase enzyme system before producing pulmonary neoplasms, knowledge of the distribution of AHH activity within the tracheobronchial tree may help explain why pulmonary neoplasms occur at particular sites in the respiratory system. The overall pulmonary AHH activity is about 3% of human and 10% of rodent hepatic AHH activity (Autrup et al., 1981). However, the pulmonary AHH activity appears to reside in certain cell lines. Boyd showed that the
pulmonary non-ciliated bronchiolar (Clara) cell in rodents possesses high AHH activity (Boyd, 1977). The rat alveolar type II cell is also capable of metabolizing BP (Jones et al., 1982). Others (Harris et al., 1978) have presented evidence that human pulmonary alveolar macrophages can metabolize BP to the ultimate carcinogen and then release this product. Because the majority of human bronchogenic squamous cell carcinomas form in the bronchus, it is of particular relevance that human bronchial epithelium can metabolize BP (Prough et al., 1979, Autrup et al., 1980, Grover et al., 1976, and Harris et al., 1982).

Animal Models of Lung Cancer

Animal models of bronchogenic carcinomas can be produced, provided the carcinogen is held in contact with the epithelium for sufficient periods of time (Kuschner, 1968, and Shabad et al., 1964). Several different means of chemically inducing respiratory tract tumors in animals have been tried with varying degrees of success: inhalation studies, systemic administration of carcinogens, intratracheal instillation, tracheal explants and transplants, and implant techniques.

Inhalation techniques are valuable in environmental studies when examining potentially noxious respiratory agents, as this best approximates man's exposure. However, this is a poor means of inducing tumors, because chemical carcinogens are cleared from the respiratory system, and the tumor yields are low (Kuschner et al., 1957).

Interestingly, some chemical carcinogens can be introduced systemically and pulmonary tumors will form. For example, subcutaneous injection of diethylnitrosamine will induce respiratory tract tumors in Golden Syrian
hamsters (Stenback et al., 1973, and Reznik-Schuller, 1980). The organotropy of diethylnitrosamine for the respiratory tract is presumed to be due to the ability of pulmonary cells to take up and metabolize the nitrosamines (Reznik-Schuller, 1980). It is important to note that diethylnitrosamine can also induce liver tumors (Pitot et al., 1978). The problem with these animal models is that the tumors may not be localized exclusively to the respiratory system nor localized within the respiratory tree, and it is difficult to control the dose of carcinogen reaching the respiratory system.

Intratracheal instillation is a popular method of chemically inducing respiratory tract tumors. Saffiotti produced an animal model of lung cancer by instilling a saline suspension of BP adsorbed to hematite (ferric oxide) into the tracheobronchial tree of Golden Syrian hamsters (Saffiotti et al., 1968). This intervention was carried out once a week for 15 weeks. He was able to bring about tracheal and bronchial carcinomas, mainly squamous cell, in a large percentage of the surviving hamsters. This model of lung cancer closely simulated the cell type, the distribution and the mode of carcinogen delivery seen in humans habitually exposed to cigarette smoke. This method has, to a large extent, remained the mainstay in chemically induced models of lung cancer. There have been many manipulations made of the same basic method, such as type of carrier dusts, vehicle of carcinogen administration, and species of rodent (Yashimoto et al., 1980, Shabad et al., 1964, Stenback et al., 1975 and Henry et al., 1973). Although this method is effective, it suffers two major disadvantages. First, the repetitive administration of the carcinogen is time consuming, and second, the carcinomas which form are not localized, hindering studies of the preneoplastic lesions.

In order to gain access to the preneoplastic lesions, Schreiber (et
al., 1975) devised a method to induce carcinomas in a circumscribed region of
the hamster trachea. He did this by using a special catheter to apply
N-nitroso-N-methylurea to the trachea, twice weekly. After 30 repeated
exposures of the carcinogen and waiting for another 20 weeks, squamous cell
carcinoma developed in 80% of the hamsters. This method lends itself to the
study of preneoplasia because it is localized, but again it is time consuming,
and the induction time is relatively long.

An ingenious method of studying localized tracheal tumors was devised
by Kendrick (et al., 1974). He excised tracheas, flushed them with culture
media, and grafted the donor trachea into the deep subcutaneous tissue of
isogenic hosts, in rats, mice and hamsters. After a 4 week recovery, BP or
3-methycholanthrene, in gelatin plugs, was inserted into the tracheal lumen of
the graft. Although this method is reasonably effective, it certainly is not
technically feasible for large scale studies. More recently, tracheal explant
models have become available (Chopra and Cooney, 1985, and Moossman et al.,
1984) which enable investigators to study chemically induced basal cell
hyperplasia and squamous metaplasia in culture conditions, but carcinomas have
not been produced by this method.

Implant techniques of inducing respiratory tract tumors are
preferable because the administration of the carcinogen is accomplished in one
operative procedure, and thus the animals do not have to be repetitively
anesthetized and manipulated. They also induce localized tumors. As early as
1937, Andervont induced lung tumors in 10 to 20% of mice that had
dibenz(a,h)anthracene-coated threads inserted into their chest cavities.
Other investigators (Shors et al., 1978 & 1980) incorporated BP into a
cylindrical plug of silicone rubber; this carcinogen-laden plug was then
secured in the bronchial lumen of Golden Syrian hamsters. Bronchogenic carcinomas, representative of the cell types seen in humans, were produced at the site of the implant in the hamsters. Nevertheless, these implant techniques are either technically difficult and time consuming or are associated with relatively high rates of morbidity and mortality.

In summary, animal models of lung cancer have been developed, but there are several shortcomings in the existing models. First and foremost, most animal models require a labour intensive protocol to initiate and maintain the delivery of the carcinogen to the respiratory epithelium. Furthermore, most models do not produce localized carcinomas; therefore, the preneoplastic and early neoplastic lesions are difficult to study, because they are difficult to locate. The models which produce localized tumors are technically cumbersome and not suitable for large scale studies. These and other disadvantages of the currently available models prompted our search for a better model. The objective of this study was to develop a practical and reliable model of localized bronchogenic carcinoma in laboratory rodents, so that one may investigate the neoplastic process and interventions - which influence respiratory tract carcinogenesis.
MATERIALS & METHODS

Animals

Female Camm-Hartley guinea pigs (Cavia porcellus), weighing 350 to 600 g, were used in this study. They were fed Purina Guinea Pig Chow #5025 ad libitum (Ralston Purina Co., St. Louis, Missouri). Their drinking water was supplemented with ascorbic acid, approximately 0.75 g/l. Female Golden Syrian hamsters (Mesocricetus auratus), weighing 90 to 125 g, were also used. These animals were fed Purina Rodent Laboratory Chow #5001 ad libitum. Both rodents were obtained from the Charles River Breeding Laboratory Incorporation, Willington, Massachusetts. All animals were kept in metal cages with mesh floors over rock salt; the cages were steam cleaned at least twice a week. Four to six guinea pigs were housed per cage; the hamsters resided in individual cages.

Threads

Benzo(a)pyrene (3,4-benzopyrene) was obtained from Aldrich Chemical Co. Inc., Milwaukee, Wisconsin (catalogue #B1,008-0) and Sigma Chemical Co., St. Louis, Missouri (catalogue #B-1760). The benzo(a)pyrene (BP) was 98% pure, with a melting point of 175-177°C. Small amounts of BP were placed in a 1cm x 1cm x 0.5cm milled cavity in an aluminum block (Figure 1) which was placed in an oven at 190-210°C. Cotton threads, secured to an applicator device, absorbed the molten BP for 5 to 15 minutes in the oven. At least twenty threads could be impregnated with BP in one hour. Large diameter (3.0) and small diameter (5.0) cotton threads were obtained from Ethicon Suture Ltd., Peterborough, Ontario (catalogue #G862H and C182H respectively).
Some threads were coated with a thin silicone rubber sheath (SRS), composed of 100 parts Silastic 382 medical grade elastomer to one part stannous octoate catalyst, obtained from Dow Corning Corporation, Midland, Michigan. This was done by slowly dragging and twisting BP-impregnated and non-impregnated threads through a pool of catalyzing silicone rubber on disposable glass slides (Figure 2); the threads were allowed to dry, hanging freely (Figure 3), to ensure that the SRS remained thin and smooth. A small 3/8 circle cutting needle was tied to the prepared threads and transferred to 12 x 75 mm glass test tubes for steam sterilization.

The amount of potential carcinogen exposure in the animals was ascertained gravimetrically by weighing similarly oven treated cotton threads, one impregnated with the carcinogen, and one not impregnated with carcinogen. The length of the thread sewn into the trachea was also recorded.

**Surgical Procedures**

The animals fasted for 24 hours prior to the operation. The guinea pigs were anesthetized intraperitoneally with 30 to 35 mg/kg of Somnotol (sodium pentobarbital), obtained from MTC Pharmaceuticals, Hamilton, Ontario. The hamsters received 65 to 90 mg/kg of intraperitoneal Somnotol. With the rodent secured to the operation board, the ventral neck was shaved and swabbed with the antibacterial skin cleanser, Hibitane (Ayerst Laboratories, Montreal, Quebec). Using sterile drapes and blades, and wearing gloves and masks, a 2.0 to 3.0 cm long midline skin incision was made over the central portion of the ventral neck. If required, 0.1-0.5 ml of 2% xylocaine (lidocaine hydrochloride), from Astra Pharmaceuticals, Mississauga, Ontario, was injected locally. The underlying subcutaneous tissue and fascia were teased apart, and the two strap (sternohyoid) muscles were separated to expose the trachea.
(Figure 4). In the mid-third of the trachea, a thread was sewn around 4 cartilage rings in the ventral wall, in the sagittal plane, and tied on the exterior surface of the trachea (Figures 4 and 5). The wound was irrigated with sterile saline and the skin incision was closed with three to five stitches of 6.0 silk suture. The lesion was then rinsed with 3% hydrogen peroxide. Up to four guinea pigs and six hamsters could be successfully operated on in one hour.

Experiments

The first experiment consisted of 94 female Camm-Hartley guinea pigs. Of the 48 experimental guinea pigs, 20 received BP-impregnated cotton threads and 28 received BP-impregnated cotton threads with a SRS. Of the 46 control guinea pigs, 19 received non-impregnated cotton threads and 27 received non-impregnated cotton threads with a SRS.

The second experiment consisted of 70 female Golden Syrian hamsters. Of the 54 experimental hamsters, 17 received BP-impregnated cotton threads, 37 received BP-impregnated cotton threads with a SRS. Of the 16 control hamsters, 7 received non-impregnated cotton threads and 9 received non-impregnated cotton threads with a SRS.

Preparation of the Tracheas

At autopsy, the tracheas were removed and fixed for 4 to 24 hours in 2.5% glutaraldehyde made up in 0.1M sodium cacodylate buffer (pH 7.3). After glutaraldehyde fixation, the trachea was cut sagittally, in the plane of the thread. The fixed trachea was stored in 0.1M cacodylate buffer (pH 7.3), dehydrated through ascending grades of alcohol and finally embedded in glycol-
methacrylate (JB-4 Embedding Kit, Polyscience Inc., Warrington, Pennsylvania). The embedded trachea was sectioned at 2.0 to 3.5 microns on a Sorvall JB-4 microtome. A minimum of 8 slides, at 15 to 30 micron increments, were prepared on at least one half of the trachea near the thread insertion site. Usually 12 to 16 slides were prepared from each half of the trachea. These tracheal slides were stained in Harris' Hematoxylin for 10 to 20 minutes at 60 to 70°C, washed in running tap water for 10 minutes, partially dehydrated in ethanol, counterstained in 0.5% eosin for 2 to 5 minutes at room temperature, and decolourized by quick dips in absolute ethanol followed by acetone.

After immersion fixation in 2.5% glutaraldehyde, tumor specimens for TEM were post-fixed in 1% osmium tetroxide, stained en bloc with saturated uranyl acetate and embedded in Spurr's low-viscosity embedding media (Kit #1916, Polyscience Inc., Warington, Pennsylvania) as previously described (Hulbert et al., 1981). Sections were cut with glass knives on a Reichert Ultracut ultramicrotome, mounted on 100 mesh copper grids, stained with uranyl acetate and lead citrate (Hulbert et al., 1981) and observed with a Philips 400 TEM. Four blocks of hamster SCT were observed for the presence or absence of desmosomes and tonofilaments by TEM. The four hamster tracheas had been exposed to BP for 88, 91, 119 and 215 days.

**Evaluation of the Tracheas**

To be confident that BP exposed epithelium was assessed, one slide of the trachea that showed remnants of the threads was selected for each animal. These slides were then interpreted independently by two pathologists (observers A & B) in a single blind fashion. The most advanced histopathologic lesion observed was recorded from a continuum of epithelial
categories: mature ciliated respiratory epithelium (ME), squamous metaplasia (SM), three progressive degrees of intraepithelial neoplasia (IEN) mild comparable to mild epithelial dysplasia, moderate comparable to moderate epithelial dysplasia, and marked comparable to marked epithelial dysplasia and carcinoma in situ), and squamous cell carcinoma (CA). The tracheal stroma (submucosa, cartilaginous layer and adventitia) was further assessed for the presence or absence of a spindle cell tumor (SCT).

The overall interobserver error between the two pathologist's grading of the tracheal epithelium was assessed by constructing a 2-sided contingency table and determining the degree of correlation between the observers on 6 epithelial categories. The corrected (Yates correction) contingency coefficient was calculated.

The efficacy of the different thread options to induce squamous cell carcinoma in hamsters was also evaluated. To determine if the SRS significantly increased the tumor yield, the fraction of hamsters developing carcinoma with a BP-impregnated thread was compared to the fraction developing carcinoma with a BP-impregnated thread with a SRS.

The author graded the degree of epithelial inflammation and recorded the presence or absence of keratinization, in all rodent tracheas.

Liver, Lungs and Lymph Nodes

Samples of liver, lung, and ventral neck lymph nodes (only in hamsters) were fixed in 10% formalin, prepared by routine histologic methods (paraffin wax), sectioned at 7 microns and stained with Harris's Hematoxylin and Eosin.
The histopathology of the livers was assessed in 10 control guinea pigs (ranging from 55 to 298 days post-operation) and in 12 experimental guinea pigs (ranging from 83 to 265 days post-operation). The histopathology of the lungs was assessed in 14 control guinea pigs (ranging from 55 to 348 days post-operation) and 19 experimental guinea pigs (ranging from 15 to 342 days post-operation). The upper and lower lobes of both the right and left lung were evaluated.

Similarly, the livers were assessed in 12 control hamsters (ranging from 1 to 250 days post-operation) and 11 experimental hamsters (ranging from 31 to 209 days post-operation). Twenty-five hamster lungs were evaluated: 15 controls (ranging from 1 to 250 days post-operation) and 10 experimental animals (ranging from 31 to 180 days post-operation). In addition, the lymph nodes were assessed in 21 experimental hamsters (ranging from 84 to 209 days) and in 5 control hamsters (ranging from 34 to 210 days post-operation).

Animal Survival

Animals that remained healthy were sacrificed at pre-determined times for morphology by pentobarbital overdose, and the animals that developed stridor were sacrificed when it became severe. Some animals died spontaneously. The mode and date of death were recorded for all animals and constituted part of the survival data. The hamsters either died or were sacrificed within 250 days of the operation and the guinea pigs within 350 days.
RESULTS

Benzo(a)pyrene Content of the Threads

Cotton threads were used because they are non-absorbable and were found to absorb considerably more BP than nylon, silk and other synthetic suture threads. The 5.0 cotton thread absorbed an average of 106 ug of BP per cm of thread (range of 44 to 155 ug/cm); whereas, the 3.0 cotton thread absorbed an average of 382 ug of BP per cm of thread (range of 145 to 865 ug/cm). In the hamsters, an average of 1.4 cm of thread was inserted into the trachea (range of 1.3 to 1.6 cm), and the guinea pigs received an average of 2.1 cm of thread (range of 2.0 to 2.2 cm). The external diameter of a 5.0 thread with a SRS approximated that of a 3.0 thread without a SRS.

Histopathology of the Tracheas

Figure 5 shows the course of the thread in the ventral tracheal wall. Note that the thread encompassed all layers of the trachea: mucosa, submucosa, cartilaginous layer and adventitia. A low power photomicrograph of a thread within the tracheal wall of an experimental hamster (Figure 6) is provided for orientation. The epithelium (indicated by arrowheads) can be seen along the surface of the tracheal lumen (TL) and also extends down into the stroma(S) along the thread (TD). Areas "B" and "C" are where the proliferating epithelium was in close contact with the thread; the most advanced epithelial changes were seen in this area, and as such, this is the epithelium which was assessed in the study. Most of the subsequent photomicrographs are of portions of areas "B" and "C", especially the site where the thread pierced through the tracheal wall into the lumen. Area "A"
serves as an internal control, as it did not undergo much change other than epithelial hyperplasia and inflammation.

**Histopathology of the Tracheal Epithelium in the Control Animals**

The cellular response of guinea pig trachea to non-impregnated threads (the control animals) is shown in Figures 7 through 11. Within 4 days of the operation, the tracheal epithelium adjacent to the thread showed a regenerative basal cell hyperplasia (Figures 7 & 8). The normal ciliated pseudostratified columnar tracheal epithelium (refer to Figure 12) has been insulted by the operation. In response, the basal cells proliferate to repair the damaged and denuded epithelium. Arrows indicate cells undergoing mitosis. The epithelium was also moderately infiltrated with acute inflammatory cells. Figure 9 shows an attenuated layer of regenerating squamous epithelium beginning to cover the thread (TD) within the tracheal lumen (TL), at 20 days post-operation. By 6 months the tracheal wound was completely healed in the control guinea pigs, and the mature tracheal epithelium was reconstituted (Figures 10 & 11). The cotton thread (TD) was not resorbed. It remained in the tracheal stroma sheathed by giant cells, macrophages and collagen (F). The same sequence of cellular changes were seen in the hamster controls, but in general, the lesions healed in about half the time required for the guinea pigs.

**Histopathology of the Tracheal Epithelium in the Experimental Animals**

The histopathologic response of the trachea to BP-impregnated threads was much different than to the non-impregnated threads. In the experimental animals, the regenerating epithelium grew along the thread into the stroma
considerably more than in the control animals, and the thread within the tracheal lumen was usually only partially re-epithelized. The tracheal epithelium showed a progressive increase in the degree of intraepithelial neoplasia, which culminates, in many of the experimental animals, as squamous cell carcinoma.

The orderly sequence of mature respiratory epithelium (ME), to squamous metaplasia (SM), to mild, moderate and marked intraepithelial neoplasia (IEN) is shown in Figures 12 to 17. These figures served as the standards from which the observers graded the tracheal epithelium in all animals in the study. Figure 12 shows the morphologic appearance of normal guinea pig tracheal epithelium. This ciliated pseudostratified columnar epithelium consists of three cell types: basal cells (arrowheads), ciliated columnar cells (curved arrows), and goblet cells (arrowheads). The normal hamster tracheal epithelium was similar; although, the hamster epithelium tended to be slightly flatter and the columnar cells more cuboidal. The subsequent epithelial categories of squamous metaplasia and intraepithelial neoplasia were virtually identical in the two species of animals. Figure 13 shows the polarized basal cells (arrowheads) and the stratified squamous epithelium (arrows), typical of squamous metaplasia. This was an early finding in both the experimental and control animals. The squamous metaplasia eventually reverted back to normal tracheal epithelium in the controls (Figure 10 and 11). But with increasing exposure to BP, the experimental animals showed progressively more dysplasia within this squamous metaplastic epithelium. Figure 14 shows an example of mild intraepithelial neoplasia. The basal cells (arrowheads) are reasonably well polarized, but there are a few immature epithelial cells (arrows) in the superficial epithelium. As the basal cells
lose their orderly arrangement along the basement membrane and as more immature epithelial cells move to the superficial layer of the epithelium, the designation of moderate intraepithelial neoplasia is warranted (Figure 15). Figure 16 & 17 show examples of marked intraepithelial neoplasia; there are immature epithelial cells throughout the height of the epithelium. Numerous atypical epithelial cells (arrows), as well as immature epithelial cells, and marked undulation of the basal aspect of the epithelium are evident in Figure 17. This photomicrograph represents carcinoma in-situ which is believed to be the precursor to invasive squamous cell carcinoma. We did not specifically grade for carcinoma in-situ, but instead included it within the confines of the broader category of marked intraepithelial neoplasia.

Figures 18 to 21 illustrate the resultant squamous cell carcinomas (CA). An example of a locally invasive squamous cell carcinoma, in a guinea pig exposed to BP for 265 days, is shown in Figures 18 & 19. The cellular features of a moderately differentiated squamous cell carcinoma are evident in Figure 19: prominent nucleoli, pleomorphism (variation in cellular shape), anisocytosis (variation in cellular size) and mitotic figures (the presence of which usually suggests increased rate of cell division). Figure 20 shows a well differentiated squamous cell carcinoma with tongues of malignant epithelium projecting into the underlying stroma, in a hamster exposed to BP for 139 days. The overall morphologic pattern of the sole guinea pig squamous cell carcinoma (Figure 18 & 19) and this hamster squamous cell carcinoma (Figure 20) are similar. However, in contrast to these two classic examples of squamous cell carcinoma, a slightly different microscopic pattern was seen in the majority of the hamster tumors.
Spindle Cell Tumors

The majority of the experimental hamsters that developed squamous cell carcinomas also developed spindle cell tumors in the tracheal stroma. Figures 21, 22 and 23 are microscopic and gross photographs of an experimental hamster after exposure to BP for 91 days. The low power light microscopic photomicrograph (Figure 21) shows the two tumor components: the superficial microinvasive squamous cell carcinoma (CA) and the stromal spindle cell tumor (SCT). In several instances, the malignant spindle cells appeared to bud off from the invading tongues of squamous cell carcinoma. With a high power view (Figure 22), the cellular features of the anaplastic spindle cell tumor are evident. The cells contained abnormal nuclei with prominent nucleoli, marked irregularity of the nucleus and many mitotic figures. The cells had an abundant cytoplasm. Although, the majority of the cells were spindle or fusiform shaped, much variability existed in cellular shape (pleomorphism), and size (anisiocytosis). The only additional feature that electron microscopy revealed was that the spindle cells were filled with markedly dilated rough endoplasmic reticulum. The search for true desmosomes between adjacent malignant spindle cells was not successful, but pseudodesmosomes were found: the cytoplasmic plaques were distinctive, but evidence of the intermediate line and associated tonofilaments was not seen.

The anaplastic spindle cells comprised the majority of the tumor mass on the trachea of the experimental hamsters (Figure 23). Within the primary tracheal tumor, the spindle cells were often found invading the adjacent skeletal muscle. A metastatic spindle cell tumor was found as a subcutaneous lump in the dorsal neck of one hamster (same hamster as in Figures 21, 22 and 23). The metastatic tumor plus the local invasion document the malignant
potential of the spindle cell tumor.

The tracheal cartilage rings encompassed by the thread underwent remodeling in all of the animals. In the experimental hamsters, ectopic foci of abnormal cartilage (chondroid metaplasia in the SCT) emerged occasionally and one presumed chondrosarcoma was identified.

Evaluation of the Histopathology of the Tracheal Epithelium

Tracheal sections were not available on all rodents. The thread could not be found at the time of autopsy in 5 experimental and 3 control guinea pigs, and thus, these tracheas were not evaluated; 3 experimental and 7 control guinea pigs died within 2 days of the operation due to pentobarbital overdose or improperly inserted threads, and these tracheas were not processed. In the hamsters, the thread could not be found in 3 experimental tracheas; one experimental trachea was lost for study because the animal died, and the trachea was autolyzed; no control tracheas were deleted. This means that the tracheas of 40/48 experimental and 36/46 control guinea pigs, and 50/54 experimental and 16/16 control hamsters were available for histopathologic observation.

Table 1 shows the results of the assessment of the overall interobserver error between two pathologists in their histopathologic grading of the tracheal epithelium. This contingency table includes the morphologic data from control and experimental animals of both species, a total of 142 observations (76 guinea pigs and 66 hamsters). The overall corrected contingency coefficient was 82%. The upper left to lower right diagonal contains 77/142 (52%) observations (dashed line), where there was perfect correlation between the two observers. To be conservative, it was decided
that a difference of one epithelial category between observers would be accepted, and that the more benign lesion of the two categories would be assigned. This range included 120/142 (85%) of the data (data within the solid lines). The remaining 15% (22/142) of the data was not included in the subsequent analysis because the two pathologists differed in their interpretations of a given tracheal slide by two or more epithelial categories. This left 35 control and 35 experimental guinea pigs and 15 control and 35 experimental hamsters for the final analysis.

Figures 24 & 25 show the histopathologic grade assigned to the tracheal epithelium of each rodent when sacrificed at the indicated days after surgery. Only mature epithelium or squamous metaplasia was seen in the 35 control guinea pigs. Two carcinomas were reported in the 35 experimental guinea pigs, one at 54 and one at 265 days post-operation. On retrospective analysis, the squamous cell carcinoma at 54 days in the experimental guinea pig proved not to be a true carcinoma (see discussion). Mild or moderate IEN was observed in 17/35 (49%) of the experimental guinea pigs, while ME and SM was reported in the remaining 16/35 (46%) of the experimental guinea pigs.

In the control hamsters, the mature respiratory epithelium was reconstituted by 80 days post-operation. The carcinoma reported in the control hamster was spurious (see discussion). On the other hand, 11/35 (31%) of the experimental hamsters demonstrated IEN. The first squamous cell carcinoma was seen at 55 days post-operation, another at 82 days and several thereafter. Overall, 15/35 (43%) of the experimental hamsters had developed squamous cell carcinoma by the end of the study. Spindle cell tumors (SCT) were also plotted as a function of time (Figure 25); 15/35 (43%) of the experimental hamsters developed a spindle cell tumor.
Efficacy of Different Thread Options to Induce Squamous Cell Carcinoma

Table 2 presents the data evaluating the efficacy of the different thread options to induce squamous cell carcinomas in the hamsters. The experimental hamsters that had a thread with a silicone rubber sheath (SRS) developed carcinomas in 13/25 (52%) animals; only 2/10 (20%) of the experimental hamsters with threads lacking the SRS developed carcinomas. Furthermore, it is of interest that 1/23 (4%) of the experimental guinea pigs containing a thread with a SRS developed carcinoma; whereas, 0/12 of the experimental guinea pigs that had a thread lacking the SRS developed carcinoma.

Inflammation and Keratinization of the Tracheal Epithelium

In the guinea pigs, the epithelial inflammation was comparable for both experimental and control animals. At the end of the study, 50% of the guinea pigs showed mild epithelial inflammation, 30% moderate inflammation, 5% marked inflammation. In the hamsters, there was considerably more epithelial inflammation in the experimental than in the control animals. In the experimental hamsters, 55% showed mild epithelial inflammation, 20% moderate inflammation and 25% no inflammation. Whereas in the control hamsters, 15% show mild epithelial inflammation and the remaining 85% showed no inflammation. The epithelial inflammation was not specifically graded for the proportions of acute and chronic inflammatory cells. But the overall impression was that the hamsters tended to have more acute inflammatory cells within the epithelium than did the guinea pigs. Early in the study, the stroma in both animals was infiltrated with acute inflammatory cells. But later on, chronic inflammatory cells were more prominent, especially in the form of a foreign body response to the thread.
Keratinization of the epithelium was a prominent finding in the experimental hamsters; 23/50 (46%) of the hamsters demonstrated keratinization. Conversely, only 3/48 (6%) of the experimental guinea pigs showed evidence of keratinization. Neither the hamster nor the guinea pig controls showed any keratinization. The keratinization was usually found in the superficial layers of epithelium (Figure 13) but some intraepithelial keratinization (Figure 17) was noted, particularly the keratin pearls in the nests of squamous cell carcinoma. Hyperkeratinization and parakeratosis was often found in the tracheal epithelium of the experimental hamsters.

Complications of the Model

One of the main complications of this model is that in a small percentage of animals, the threads were extruded into the tracheal lumen. Ten percent (5/48) of the experimental guinea pigs extruded their threads by the end of the study versus seven percent (3/46) of the controls. Six percent (3/54) of the experimental hamsters extruded their threads while all (16) threads remained intact in the controls.

An abscess formed around the thread within the tracheal wall in one animal, mimicking a tumor grossly. Threads not tied firmly around the cartilage rings hung lax in the lumen, causing obstruction of the trachea and premature demise of the rodents.

Lymph Nodes, Liver and Lung

The ventral neck lymph nodes from 5/5 control hamsters were normal, as were the 8/21 lymph nodes from the experimental hamsters. The other 13/21 lymph nodes in the experimental hamsters showed plasma cell hyperplasia, but
there was no metastatic carcinoma present. A few of these lymph nodes contained distinct foreign body granulomas; the nature of the inciting agent is not known, but calcified debris, consistent with thread, was found in some granulomas.

In the control guinea pig livers, 4/10 livers were normal; 5/10 showed diffuse fatty changes; 1/10 showed evidence of mild periportal damage (hepatocyte dropout and fibrosis around the portal triad). Slightly more damage was noted in the livers from the experimental guinea pigs: 4/12 livers appeared normal, while the remaining 8/12 livers demonstrated fatty changes and mild periportal damage. Interestingly, 3/12 livers from the experimental animals showed islands of hematopoietic tissue in the liver sinusoids (extramedullary hematopoiesis).

In the hamster controls, 4/12 livers were normal while the remaining 8/12 showed fatty changes and mild periportal damage. The same spectrum of changes was observed in 11 of the experimental hamster livers, but the periportal damage was greater than in the controls.

In the lungs from the control guinea pigs, 3/14 showed histopathologic evidence of mild bronchopneumonia, compared to 8/19 of the experimental guinea pigs. The remaining lungs, 11/14 of the control and 11/19 of the experimental guinea pigs, were normal.

All 15 lungs from control hamsters were normal. One of 10 lungs from the experimental hamsters showed a focal bronchopneumonia, likely due to aspiration during the initial operation, as foreign body giant cells were also present. Curiously, one experimental hamster showed an intrapulmonary vasculitis, without other evidence of parenchymal damage.
Animal Survival

The overall cumulative morbidity and mortality in the guinea pigs and hamsters is presented in Figures 26 & 27. As would be expected, the control animals survived longer than the experimental animals. By the end of the study, 35% of the control guinea pigs had either died spontaneously or became sick enough that they were sacrificed, as opposed to 70% for the experimental guinea pigs. The same overall figures were observed in the hamsters. For both the guinea pig and hamster controls, the morbidity and mortality does not increase much after 150 to 100 days, respectively. However, both the experimental rodents undergo an increase in morbidity and mortality with time, that levels off in the latter third of the study period.
Figure 1  Impregnation of cotton threads with benzo(a)pyrene (BP). Cotton threads secured to applicator device (arrow) were dipped into the aluminum block containing molten BP.

Figure 2  Preparation of the silicone rubber sheath (SRS). Cotton threads were slowly dragged through a puddle of catalyzing silicone rubber (arrow).

Figure 3  Enlarged view of apparatus used to coat cotton threads with a SRS. Coated threads were allowed to dry hanging freely (arrow).
Figure 4  Diagram of the surgical procedure performed on the rodents. After intraperitoneal anesthesia, a midline skin incision was made. The underlying subcutaneous tissue and fascia was teased apart, and the two strap muscles (arrow heads) were separated to expose the trachea. In the mid-third of the trachea, a thread (arrow) was sewn around 4 cartilage rings in the ventral wall and tied on the exterior surface of the trachea.

Figure 5  Diagram of a tumor (arrowhead) forming around the thread (arrow) within the ventral tracheal wall. The thread encompassed all layers of the trachea: mucosa, submucosa, cartilaginous layer and adventitia.

Figure 6  Low power photomicrograph of a thread within the tracheal wall of an experimental hamster. Areas "B" and "C" are where the proliferating epithelium was in close contact with the thread; the most advanced epithelial changes were seen in this area, and as such, this is the epithelium which was assessed in the study. The subsequent photomicrographs are of portions of areas "B" and "C". Area "A" served as an internal control; for the most part, it did not undergo much change other than epithelial hyperplasia and inflammation. The thread (TD) can be seen wrapped around the cartilage rings (arrows). The epithelium is indicated by arrowheads, and an "S" marks the tracheal stroma (submucosa, cartilagenous layer and adventitia). The tracheal lumen (TL) separates the dorsal wall (area "A") from the ventral wall (area "B" and "C") x 25.
PLATE III

Figure 7  Epithelium adjacent to the site where the thread pierces into the tracheal lumen (TL), in a guinea pig control, 4 days post-operation. An arrow marks a cell undergoing mitosis in the proliferating epithelium. The epithelium is moderately inflamed, as is the underlying stroma (S) x 172.

Figure 8  A high power view of cells in the aforementioned tracheal epithelium (Figure 7) undergoing mitosis (arrows), documenting the rampant cellular proliferation. Acute inflammatory cells (arrowheads) are found within the epithelium x 440.

Figure 9  An attenuated layer of regenerating squamous epithelium (arrow) covers the thread (TD) within the tracheal lumen (TL), guinea pig control, 20 days post-operation. Adjacent to the thread is relatively mature respiratory epithelium (arrowhead) which overlies a moderately inflamed stroma (S) x 172.

Figure 10  Low power view of a trachea in a guinea pig control, 183 days post-operation. The mature respiratory epithelium (arrowhead) is completely reconstituted. Note the thread (TD) in submucosa, the tracheal cartilage ring (C) and the attendant fibrosis (F) around the thread x 70.

Figure 11  High power view of mature tracheal epithelium (arrowhead), corresponding to the aforementioned low power photomicrograph (Figure 10). Chronic inflammatory cells are seen in the submucosa. Fibrosis (F) around a thread remnant is present x 172.
Figure 12 High power view of normal guinea pig tracheal epithelium (hamster tracheal epithelium is similar). This ciliated pseudostratified columnar epithelium consists of three cell types: basal cells (arrowheads), ciliated columnar cells (curved arrows) and goblet cells (straight arrows) x 440.

Figure 13 Squamous metaplasia (SM) with keratinization (K) in an experimental hamster. The polarized basal cells (arrowhead) lie beneath the superficial squamous cells (arrow). A cartilage ring (C) is marked x 172.

Figure 14 Mild intraepithelial neoplasia (mild epithelial dysplasia) in an experimental hamster. Again the basal cells (arrowheads) are reasonably well polarized, but there are a few immature cells (arrows) in the superficial epithelium x 172.

Figure 15 Moderate intraepithelial neoplasia (moderate epithelial dysplasia) in an experimental hamster. The basal cells (arrowheads) are now poorly polarized, and immature cells (arrow) are found in superficial layers of the epithelium. The underlying cartilage (C) is indicated x 172.

Figure 16 Marked intraepithelial neoplasia (marked epithelial dysplasia) in an experimental hamster. The basal cells (arrowhead) are indicated. Immature cells (arrows) can be found throughout the epithelium x 172.
Figure 17  Marked intraepithelial neoplasia (carcinoma in situ) in an experimental hamster. The basal aspect of the epithelium is undulated (arrowhead), and the malignant epithelial cells are ready to break the confines of the basement membrane. Atypical cells (arrows) are present. Keratinization is evident, both at the epithelial surface (K) and intraepithelial (IEK) x 172.
PLATE V

Figure 18  Low power view of nests of malignant squamous cell carcinoma (CA) invading the tracheal wall in a guinea pig exposed to BP for 265 days x 70.

Figure 19  High power view of Figure 18 illustrating the squamous cell carcinoma in the guinea pig trachea. The pleomorphism (variation in cellular shape), anisocytosis (variation in cellular size) and mitotic figures (arrow) typical of malignant epithelium are evident in this photomicrograph x 440.

Figure 20  Well differentiated squamous cell carcinoma (CA) in a hamster exposed to BP for 139 days x 172.

Figure 21  A photomicrograph of a superficial microinvasive squamous cell carcinoma (CA), and accompanying spindle cell tumor (SCT) in the stroma. The two tumor components appear to merge (arrows). Figures 21, 22 and 23 are of the same hamster, exposed to BP for 91 days x 172.

Figure 22  High power view of Figure 21 showing the spindle cell tumor. Several mitotic figures (arrows) are indicated, amongst the other anaplastic spindle cells x 440.

Figure 23  Gross appearance of a tracheal tumor (TU) in a hamster.
**TABLE I**

**ASSESSMENT OF THE OVERALL INTEROBSERVER ERROR IN THE HISTOPATHOLOGICAL GRADING OF THE TRACHEAL EPITHELIUM**

**OBSERVER A**

<table>
<thead>
<tr>
<th></th>
<th>ME</th>
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<th>IEN₂</th>
<th>IEN₃</th>
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**STATISTICS:** Corrected contingency coefficient = 82% (a perfect correlation is 100%)

These data represent the histopathologic observation of the tracheal epithelium by two pathologists (observer A & B) on a total of 142 rodents (76 guinea pigs and 66 hamsters). Each datum denotes the number of corresponding histopathologic observations; the upper left to lower right diagonal of data is the number of perfect histopathologic correlations between the observers. The data within the solid lines are the acceptable morphologic observations.
Figure 24  Histopathologic data of guinea pig tracheal epithelium: controls (dashed line) and experimental (solid line) animals. Each point represents a rodent sacrificed at the indicated time. Only those morphologic interpretations with an acceptable interobserver error are included. (35 control and 35 experimental guinea pigs). The carcinoma (CA) reported in the experimental guinea pig at 54 days was not a CA; it was a pseudoepithelomatous hyperplasia, with mild intraepithelial neoplasia.

Figure 25  Histopathologic data of hamster tracheal epithelium: controls (dashed line) and experimental (solid line) animals. Each point represents a rodent sacrificed at the indicated time. Only those morphologic interpretations with an acceptable interobserver error are included. (15 control and 35 experimental hamsters). The hamsters that developed a spindle cell tumor (SCT) in the stroma are denoted above by an "x" at the indicated sacrifice times; only data points with perfect interobserver correlation are included (15 experimental hamsters). The carcinoma (CA) reported in a control hamster at 20 days was not a CA; it was a coalescing group of activated macrophages (epitheloid cells) in the stroma which appeared to arise from the overlying metaplastic squamous epithelium.
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# of hamsters not developing CA

|                  | 7                | 8                      | 8                      | 12                     |

# of hamsters developing CA

|                  | 0                | 1                      | 2                      | 13                     |

Only the number of squamous cell carcinomas (CA) which both observers independently agreed upon are included in this data. The one carcinoma reported in the controls was not a carcinoma (see legend for Figure 25). The presence of the silicone rubber sheath (SRS) appears to increase the efficacy of inducing squamous cell carcinomas in hamsters; a chi-squared analysis of C versus D is significant at $p \leq 0.10$. 
Numbering error. Text for page 46 not available.
Figure 26  The overall cumulative summed mortality and morbidity in the guinea pigs, expressed as a percentage of the initial number of guinea pigs recruited for surgery, is shown in solid bars. The cumulative percentage of animals that were healthy but sacrificed for morphology is shown in open bars. A total of 46 control (Con) and 48 experimental (Exp) guinea pigs were used in this study.

Figure 27  The overall cumulative summed mortality and morbidity in the hamsters, expressed as a percentage of the initial number of hamsters recruited for surgery, is shown in solid bars. The cumulative percentage of animals that were healthy but sacrificed for morphology is shown in open bars. A total of 16 control (Con) and 54 experimental (Exp) hamsters were used in this study.
DISCUSSION

Consideration of the Histopathologic Results

Since we were interested in developing a model of bronchogenic carcinoma which allowed us to study the epithelium at various stages in the development of the carcinoma, we purposely sacrificed animals during the preneoplastic phase, before carcinomas developed. Since grading IEN is invariably judgemental, it was necessary to construct standards (Figure 12 to 17) and to ascertain the degree of interobserver correlation between the independent interpretations of the two histopathologists. Overall, a good degree of interobserver correlation was observed (see Table I), with a corrected contingency coefficient of 82%. Excellent correlation was obtained when assessing mature epithelium, squamous metaplasia and squamous cell carcinoma. And as to be expected, there was some disparity between the two observers when reading the IEN. In order to maintain the accuracy of the histopathologic data, readings which differed by more than one epithelial category were excluded (22 of 142) from the final results. Because the greatest interobserver error was seen when grading IEN, excluding these results translates to excluding animals with IEN. The intraobserver error was not assessed.

The author spent considerable time reviewing the same slides and agreed on all the diagnosis except 2 out of 142. On a retrospective analysis, the squamous cell carcinoma at 54 days in the experimental guinea pig proved not to be a true carcinoma, but rather, pseudoepitheliomatous hyperplasia mimicking a carcinoma. The other squamous cell carcinoma in the guinea pig was real (Figures 18 & 19). Similarly, the carcinoma reported in the control
hamster at 20 days post-operation was an exuberant stromal reaction to the thread, numerous activated macrophages with an epitheloid appearance which appeared to merge with the overlying squamous metaplastic epithelium. The author's histopathologic assessment of the tracheal epithelium was excluded from the data because it was not performed blind.

The IEN emerged early, within 40 days, in both experimental animals (Figure 24 & 25). Although it was not specifically tested, it appeared that the experimental animals fitted with threads lacking the SRS developed IEN earlier than animals possessing threads with a SRS. This might be expected, because without the SRS to control the release rate, the BP would diffuse out of the thread relatively quickly to induce IEN, but the lesions could then be repaired (recall that IEN is a potentially reversible lesion). The moderate degree of IEN in the guinea pigs sacrificed at 30 to 50 days post-operation, and the lack of progression of the IEN with time reflects this notion, most of the guinea pigs in this early phase of the study were animals fitted with the large diameter (3.0) thread without a SRS.

IEN was seen in the tracheal epithelium of several experimental hamsters, sacrificed before 70 days post-operation. This is not reflected in the data presented in figure 25, as there was relatively more disagreement between the two observers when assessing these slides with IEN, and many of these data points were discarded. A few of the experimental hamsters were found to have only squamous metaplasia or mature respiratory epithelium, despite an adequate duration of exposure to BP. This might be explained by differences amongst individual animals in metabolizing BP, clearing the BP, or repairing the BP mediated cell damage. Since the amount of BP per thread varied by up to 400 percent, another possible explanation is that the
experimental animals not developing IEN or CA received threads with less BP.

When considering the yield of carcinoma in this animal model, rather than stating the number of carcinomas that developed over the entire duration of the study as a fraction of all the animals entered into the study (a cumulative measure of cancer incidence), it is preferable to calculate the number of carcinomas that develop after a minimum time for induction of cancer. In the experimental guinea pigs, a reasonable minimal time for cancer development is 200 days. There were 11 experimental guinea pigs sacrificed after 200 days and up to 350 days, with one squamous cell carcinoma - a carcinoma yield of 1/11 (9%). With the minimum cancer induction time set at 120 days in the hamsters, the carcinoma yield is 9/14 (65%). The data suggest that the hamster is the most useful animal to study bronchogenic squamous cell carcinomas; and to obtain the greatest yields, they should be sacrificed between 120 to 150 days after the operation.

Further to the low yield of carcinomas observed in the guinea pigs, the spontaneous tumor rate in guinea pigs is extremely low (Warren and Gates, 1941, and Rogers and Blumenthal, 1960). The pulmonary tumors that form spontaneously are peripheral alveolar adenoma, hemangiosarcoma, lymphangioma, intrabronchial papilloma and adenomatosis (Hoch-Ligeti et al., 1983). Guinea pigs do not develop the common histologic types of pulmonary tumors seen in humans. It is generally considered that guinea pigs are relatively resistant to experimentally induced respiratory tract tumors (Blacklock, 1961, Russel and Ortega, 1952 and Hoch-Ligeti and Argus, 1970). However, some investigators have been able to induce pulmonary tumors with oral diethylnitrosamine (Argus and Hoch-Ligeti, 1963), gavage dimethylmorpholine (Lijinsky and Reuben, 1981), and intravenous injection of
methylcholanthrene and dibenzanthracene (Heston and Deringer, 1952). Nevertheless, the tumor yields of these guinea pig models are low, and the tumors are primarily peripheral adenomas and adenomatous lesions. The one bona fide squamous cell carcinoma, which we observed in the guinea pig trachea is the only tracheobronchial squamous cell carcinoma and the only BP-induced carcinoma in guinea pigs found in reviewing the literature.

A large subset of bronchogenic carcinomas in humans are squamous cell, as are the tumors in these rodents. BP is a major carcinogen in cigarette smoke, and it is the carcinogen used in this animal model. The human bronchial epithelium can metabolize BP (Prough et al., 1979; Autrup et al., 1980; Grover et al., 1976; and Harris et al., 1982), as can the tracheal epithelium of hamsters (Eastman et al., 1981, Moore and Cohen, 1978, and Mass and Kaufman, 1978). The human bronchial versus the rodent tracheal site of tumor formation is an expected different and does not invalidate the comparison, especially when it is known that repeated intratracheal instillation of BP into hamster lungs, simulating inhalation of cigarette smoke into human lungs, produces basically the same spectrum and distribution of pulmonary tumors as in humans (Saffiotti et al., 1968). Although this animal model may be not useful to study human small or large cell carcinomas and adenocarcinomas, it certainly will be useful to study squamous cell bronchogenic carcinomas.

**Efficacy of Different Thread Options to Induce Squamous Cell Carcinoma**

Initially during the development of the model, the BP-impregnated threads were not coated with a silicone rubber sheath (SRS). Later, in an effort to increase the efficacy of inducing carcinomas, SRS were added to the
thread to decrease the diffusion rate of BP into the tissues. The half life of release of BP from this particular silicone rubber matrix is about 2 months (Shors et al., 1980). The release rate of BP from cotton threads was not measured. But since there was less impediment to diffusion, it was assumed that threads without the SRS would release the BP faster than threads with a SRS. Work with tumors induced with 7,12-dimethylbenz(a)anthracene in rat tracheas have indicated that lower doses of carcinogen delivered slowly are more effective in producing dysplastic and neoplastic lesions than higher doses delivered rapidly (Shiba et al., 1982). Presumably, the protracted lower BP exposure increases the incidence of cell initiation and thereby increases the probability of inducing a carcinoma. Whereas, a shorter duration of concentrated BP exposure is toxic to the cell and thus is less effective in inducing carcinomas. The drawback of adding a SRS to the thread is that it takes slightly longer to prepare the thread, but this appears worthwhile as the data (Table 2) do suggest that the SRS increases the yield of carcinomas.

The concentration of BP in the thread was an uncontrolled variable, as both 3.0 (large diameter) and 5.0 (small diameter) cotton threads were used in this study. The 3.0 BP-impregnated thread exposes the animals to three to four times the amount of BP as a 5.0 thread, 535 to 800 ug and 150 to 225 ug of BP respectively. The differential response of the tracheal epithelium to the different size threads (different potential BP exposures) was not determined because the groups were small in number if also subgrouped into those with and without a SRS. Nevertheless, the concentration of BP appeared to be less important than the presence of the SRS in the induction of carcinomas, because no experimental animals with a 3.0 thread (0/1 hamsters and 0/12 guinea pigs) developed CA.
Tissue Origin of the Spindle Cell Tumor

The squamous cell carcinomas which were found in the experimental animals are clearly derived from the tracheal epithelium. However, the cellular derivation of the stromal spindle cell tumor (SCT) in the hamsters is not known. The term 'spindle cell tumor' is merely descriptive; it does not imply any particular cellular origin. The SCT is not a pseudosarcoma, a stromal reaction to the presence of squamous cell carcinoma, because the SCT exhibits malignant cellular features, local invasion and in one case, metastatic disease. There are two possibilities for the tissue derivation of the SCT: the SCT may be a variant of squamous cell carcinoma, a spindle cell carcinoma or it may be derived from tracheal mesenchymal elements, a fibrosarcoma for example.

From light microscopic observations, it appeared that the SCT in the hamsters derives from the squamous cell carcinoma, and thus, it constitutes a spindle cell carcinoma. In several instances (refer to Figure 21), the stromal SCT appeared to be in continuity with the superficial squamous cell carcinoma. This apparent imperceptible merging of the two tumor components is identical to a similar photomicrograph (Figure 4, page 23) of Stinson and associates (Stinson et al., 1983) in which they unequivocally demonstrated by TEM, that a hamster tracheal SCT was a spindle cell carcinoma. The light microscopic observations suggest but do not prove that the SCT is a spindle cell carcinoma.

Ultrastructural demonstration of desmosomes and tonofilaments is often regarded as evidence for the epithelial derivation of a SCT. Yet there is no universal law in biology which states that spindle cell carcinomas must contain these cellular features in order to be considered of epithelial
origin. In fact, these cellular elements become less distinct as the epithelium moves from hyperplasia, to squamous metaplasia, to intraepithelial neoplasia, to squamous cell carcinoma (Muller and Sutherland, 1971), and it is reasonable to expect to see a relative paucity of these elements when an even more anaplastic form of a squamous cell carcinoma forms, the spindle cell carcinoma. The TEM investigation of the hamster SCT showed pseudodesmosomes, which lacked the intermediate line between the two opposing cytoplasmic plaques and the tonofilament association with the cytoplasmic plaques seen in true desmosomes. Poorly defined desmosomes are commonly found in spindle cell carcinomas (Lichtiger et al., 1970). Also it is usually necessary to cut several blocks for TEM before true desmosomes can be found (Battifora, 1976) and only one grid from each of four SCT was examined in this study. Therefore, our inability to demonstrate desmosomes and tonofilaments does not necessarily preclude the possibility that the hamster SCT is a spindle cell carcinoma.

A preliminary study conducted in an attempt to type the intermediate filaments of the SCT was inconclusive. The mouse-monoclonal antibodies used (Tissue Origin Identification Kit, #EAB-901, from Enzo Biochem Inc., New York, New York) were directed against human antigens (Gown and Vogel, 1982 and 1984). Because epithelial intermediate filaments (tonofilaments) differ somewhat between species (Lane, 1982), it was felt the mouse-monoclonal antibodies to human antigens may not have been suitable probes to demonstrate hamster tonofilaments. Antibodies to hamster tonofilaments would be ideal, failing that, a polyclonal rather than a monoclonal sera may be of some value.

TEM of the SCT revealed that the tumor cells were filled with dilated rough endoplasmic reticulum. This along with the presence of collagen in the
intercellular matrix implied that the spindle cells were actually producing collagen. This observation suggested that the SCT were of fibroblastic origin, and could be considered fibrosarcomas. However, non-neoplastic epithelium is known to produce the type IV collagen of the basal lamina (Kefalides, 1971), and corneal epithelium can secrete collagen (Hay and Dodson, 1973). Furthermore, it has also been clearly shown by Battifora (Battifora, 1976) that human spindle cell carcinomas, with well formed desmosomes, do demonstrate morphologic evidence of collagen production, as does the hamster SCT.

The tracheal cartilage rings exposed to BP underwent several changes that may provide clues to the origin of the SCT. There was at least one example of an area of chondroid metaplasia within the SCT and one example of abnormal cartilage which was presumed to be a well differentiated chondrosarcoma. The cartilage was not evaluated in all tracheal specimens, but abnormal cartilage appeared in many hamster tracheas. If one mesenchymal element (cartilage) can form a neoplasm, it might be reasonable to expect other mesenchymal elements (fibroblasts) to form neoplasms, perhaps suggesting that the SCT was a sarcoma.

In the final analysis, it is impossible to state definitively that the hamster SCT is a carcinoma or a sarcoma. The light microscopic observations suggested that the SCT is a spindle cell carcinoma, but the corroborative TEM and intermediate filament typing results were equivocal. If the SCT is in fact a sarcoma, one may have to cautiously extrapolate the results of a particular application of the hamster model to human bronchogenic carcinoma, because of the possibility that the sarcoma may modify the response
of the squamous cell carcinoma to the tested variable (eg. a therapeutic agent). On the other hand, if the SCT is a spindle cell carcinoma, the model is particularly valuable, since it allows the morphologist access to a wide spectrum of preneoplastic and neoplastic epithelium. Future studies may clarify this issue.

The Role of Inflammation, Regenerative Hyperplasia and Keratinization of the Tracheal Epithelium in Tumor Promotion

The inflammation of the tracheal epithelium was recorded because persistent inflammation may contribute to the carcinogenic process. There were considerably more acute inflammatory cells (polymorphonuclear leukocytes) in the epithelium of the experimental versus the control hamsters. Poly-morphonuclear leukocytes (PMN) in inflammatory lesions are known to produce oxygen free radicals (Babior, 1978). It has also been shown that PMN stimulated with phorbol esters generate oxygen free radicals (Goldstein et al., 1981) which cause cytogenetic changes in cultured mammalian cells (Weitberg et al., 1983). Additionally, PMN can activate BP to genotoxic metabolites via a reactive oxygen-dependent reaction (Trush et al., 1985). So the PMN in the experimental hamster tracheas may have generated sufficient oxygen free radicals to contribute to the BP-mediated DNA damage and carcinogenesis.

The surgical insult to the trachea during the operation initially incited a regenerative basal cell hyperplasia: tissue damage results in necrosis, and cell death is known to act as a mitogenic stimulus (Columbano et al., 1981, and Farber, 1981). Within one month, the hamster tracheal
epithelium resembled a hyperplastic epidermis, with a thick layer of squamous epithelium and attendant superficial keratinization. In mouse models, repeated abrasion of chemically initiated skin results in epidermal hyperplasia and sufficient tumor promotion for carcinoma formation (Argyris and Slaga, 1981 and Argyris, 1982). Again based on models of carcinoma in mouse skin, wounding can act as a tumor promoter (Pelling and Slaga, 1985 and Marks et al., 1982). It is possible in our model that the surgical insult, independent of the inflammation, may have induced a regenerative basal cell hyperplasia and that this cell proliferation contributed to the carcinogenesis, particularly to tumor promotion.

Phorbol esters, known tumor promoters, produce epidermal hyperkeratinization in mouse skin (Nelson and Slaga, 1982 and Slaga and Klein-Szanto, 1983). It is of interest that the experimental hamsters were the only group of animals which readily developed carcinomas and that keratinization (as well as inflammation) was a prominent feature of this epithelium. The relative lack of keratinization and carcinoma formation in the guinea pigs may suggest that under these experimental conditions there was insufficient tumor promotion to result in carcinomas. Perhaps something was interfering with the role of tumor promotion in the guinea pigs; this possibility will be elaborated on in the discussion of ascorbic acid and cancer.

Ascorbic Acid and Cancer

There was an obvious difference in the histologic response of hamsters and guinea pigs to the insertion of a BP-impregnated threads into the tracheas. Carcinomas formed readily in the hamsters but rarely in the guinea
pigs. The reason for this difference is not known, but several possibilities exist. First, carcinogens need to be held in contact with the epithelium for sufficient periods of time to be effective (Kushner, 1968). Perhaps guinea pigs are more efficient at clearing the liberated BP, presumably a function of macrophages. Another possibility is that the two animals possess different basal levels or degrees of inducibility of the monooxygenase enzymes necessary to convert BP to the active carcinogen (Mass and Kaufman, 1981). Alternatively, the guinea pigs may produce a less carcinogenic spectrum of BP metabolites or bring about a more effective metabolic detoxification of BP (Autrup et al., 1980, and Moore and Cohen, 1978). The two animals may differ in their capacity to repair the BP induced macromolecular damage, especially of the genome (Cohen and Ashurst, 1983, and Eastman et al., 1981). It should be stated at this point that all these possible explanations for the differences in the histologic response of hamsters versus guinea pigs are not mutually exclusive and that in fact, the difference likely reflects a complicated interrelation of these and other considerations.

Another plausible explanation for the disparity of responses to BP in the two rodents, is that the diet of the guinea pigs was supplemented with relatively high doses of ascorbic acid compared to the hamsters which, like most mammals, do not require exogenous ascorbic acid. The proposed anticancer role of ascorbic acid has long been a source of controversy. In the 1970's, Cameron and Pauling (Cameron and Campbell, 1974, Cameron and Pauling, 1976 and 1978) suggested that high dose ascorbic acid (10 grams per day) was beneficial in patients with advanced cancer, based on a retrospective comparison between selected study patients and historical control patients. Later, a
prospective, randomized, double blind clinical trial by Creagan and Moertel (Creagan et al., 1979) showed that high dose ascorbic acid therapy in patients with advanced colorectal carcinoma did not increase patient survival. Pauling argued (Pauling, 1980) that Creagan's patients had received chemotherapy which adversely interfered with the ability of ascorbic acid to enhance the immune response against the tumor (Cameron and Pauling, 1974). However, recently Moertel and Creagan (Moertel et al., 1985) repeated the study on similar patients without prior chemotherapy and reported that high dose ascorbic acid showed no advantage over placebo on disease progression or patient survival.

Although the aforementioned clinical studies suggest that ascorbic acid does not have an anticancer effect, ascorbic acid's potential anticancer role should not be dismissed prematurely. These studies assessed the effect of dietary ascorbic acid intake on tumor progression. It is possible that ascorbic acid exerts an anticancer effect at other stages in the neoplastic process, such as tumor initiation and tumor promotion. There exists in vivo (Cameron et al., 1975, Pierson and Meadows, 1983, Sanders and Mahaffey, 1983, and Varga and Airold, 1983) and in vitro (Park et al., 1980; Prasad et al., 1980, and Bram et al., 1980) evidence ascribing an anticancer role to ascorbic acid. Although the mechanisms of ascorbic acid's reputed anticancer effect are not clear, some investigators (Cerutti, 1985, and Goldstein et al., 1983) contend that oxygen free radicals can mediate tumor promotion, and that ascorbic acid is capable of scavenging oxygen free radicals.

The total dietary intake of ascorbic acid in the guinea pig was about 150 to 200 mg per kilogram body weight per day (mg/kg/day). Yet guinea pigs require only 5 mg/kg/day to grow, reproduce and survive normally (Veen-Baigent et al., 1975). Given that oxygen free radicals could be produced in our
rodent model of carcinoma by the tracheal infiltrate of PMN; that oxygen free radicals may mediate carcinogenesis; that ascorbic acid can quench oxygen free radicals, it is possible that the high supplemental dietary ascorbic acid was responsible for the observed paucity of carcinomas in the guinea pigs. This hypothesis is currently being tested in our laboratory by decreasing the dietary intake of ascorbic acid in guinea pigs and noting the subsequent frequency and chronology of carcinoma development.

Complications of the Model

This animal model of bronchogenic carcinoma is straightforward and simple, but it does have several drawbacks. The most obvious problem is tracheal obstruction, which leads to bronchopneumonia and ventilatory failure. The amount of BP delivered to the tissues is dependent on the length and diameter of impregnated threads inserted into the tracheal wall. So increasing the exposure of the trachea to BP is tantamount to increasing tracheal obstruction. Fortunately, sufficient BP (150 to 225 ug) can be delivered to the hamster tissues (with the 5.0 cotton threads) to induce carcinomas without compromising respiration.

One of the main complications of this model is that in 5 to 10 percentage of both groups of experimental animals, the thread appears to be extruded into the tracheal lumen. In order to position the inserted thread taut against the tracheal wall, the thread is tied firmly around the cartilage rings, often squeezing the cartilage rings together. Presumably because of the pressure effects of the thread and the inflammation, the cartilage rings remodel and resorb. If this process continues until the cartilage rings are completely resorbed, the thread is no longer anchored in the tracheal wall and
is extruded into the tracheal lumen. This becomes more pronounced in the late phases of the study, after the cartilage has had more time to resorb. However as the hamsters develop tumours within four to five months, the magnitude of this problem can be reduced by sacrificing the animals earlier.

An obvious drawback to the model is that the neoplasm can only undergo limited growth before ventilatory failure ensues. So by virtue of design, this model is of most utility in studying the preneoplastic lesions and early neoplastic growth. Regardless of the potential and real problems with the model, it does produce squamous cell carcinoma in a large percentage of the hamsters.

**Animal Survival**

The stated survival rates include all the animals used during the development of the model. Within this survival data, many animals are included which succumbed due to complications of the operation such as pentobarbital overdose, improperly placed threads, inadequate tension on the thread tied around the cartilage rings, excessive tracheal damage due to use of the wrong surgical needles, and other minor problems which were appreciated later in the study. These problems are reflected in the initial (0-50 days) morbidity and mortality of 10 to 30% in both animals (Figures 26 & 27). Once the thread insertion technique is mastered and the exact anaesthetic doses are determined, the overall morbidity and mortality could probably be reduced by 10 to 20%.

The guinea pigs underwent an increase in morbidity and mortality with time. This appeared to be the result of the development of bronchopneumonias, likely secondary to partial tracheal occlusion. A relatively large percentage
(approximately 40%) of the experimental guinea pigs showed histopathologic evidence of bronchopneumonias. Only 20% of the control guinea pigs developed bronchopneumonias. Despite the fact that only one experimental guinea pig developed a carcinoma, the tracheal lumen was usually more distorted in the experimental than in the control guinea pigs. These findings suggest that guinea pigs do not tolerate partial tracheal occlusion, as they are prone to develop bronchopneumonias. Thus, on this basis, they may be less suitable models of respiratory tract tumors than other rodents.

In the experimental hamsters, the progressive increase of morbidity and mortality, that levelled off towards the latter third of the study period coincided with the development of the tracheal tumors. As they encroached on the tracheal lumen, ventilatory failure ensued. It is likely the spindle cell tumor (SCT) rather than the squamous cell carcinoma (CA) component of the hamster tracheal tumor was responsible for the ventilatory failure, since the SCT constituted the majority of the tumor mass. Metastatic cancer probably did not contribute appreciably to the observed morbidity and mortality in the hamsters, because the sampled regional lymph nodes liver and lung were negative for metastatic carcinoma, and only one example of tumor metastasis was found. In humans, metastasis of bronchial carcinoma to skin or subcutaneous tissue is relatively rare in comparison to metastasis to lymph nodes, liver and lung. It is curious that a subcutaneous metastasis occurred without evidence of metastasis to other sites in any of the hamsters. Yet admittedly, a comprehensive search for tumor metastasis was not conducted.

Although inhaled BP can enter the circulatory system (Mitchell, 1982), and it is known that the liver can metabolize BP to mutagenic products
(Wood et al., 1976), we found no primary or secondary liver tumors in either rodent. Yet there was histopathologic evidence of liver damage: diffuse fatty changes and periportal hepatocyte dropout and fibrosis. This may have contributed to the observed morbidity and mortality. The liver damage was more prevalent and severe in the experimental animals, but a few control animals also showed mild liver damage. It was assumed that the liver damage was due to a cytotoxic action of BP, but the mild liver damage in the controls may suggest that a factor common to the two groups of animals, such as diet or the SRS, contributed to the observed liver damage.

The significance of the extramedullary hematopoiesis in the experimental guinea pigs is not known. It could suggest that BP insulted the bone marrow, and that the liver was recruited for hematopoiesis; however, the hematologic status of the guinea pigs was not followed throughout the course of the study.

CONCLUSION

One of the major virtues of the model is its practicality: the threads are easy to prepare; no elaborate equipment or materials are needed; the surgery is simple and quick, and the carcinogen does not need to be repetitively administered. Therefore, large scale animal studies are feasible.

Besides being readily accessible, the incipient tracheal carcinomas are marked by the thread. This enables the investigator to locate and study the preneoplastic epithelial lesions, simply by sacrificing the animal at the appropriate time. Although the definitive origin of the hamster spindle cell
tumor is unknown, it is presumed to represent a spindle cell carcinoma. Therefore, morphologists can gain access to differentiated mature respiratory epithelium, squamous metaplastic epithelium, progressive degrees of intraepithelial neoplasia, squamous cell carcinoma, and dedifferentiated anaplastic spindle cell carcinoma.

Tumors can be readily produced in the hamsters. The majority of the hamsters left to survive at least 120 days develop squamous cell carcinoma, a carcinoma incidence of 65% (9/14). By avoiding the initial complications of the operation, by sacrificing the hamsters before thread extrusion occurs, and by using only threads with a silicone rubber sheath, the carcinoma yield could probably be increased another 20%.

This model has innumerable applications. The ultrastructural alterations in epithelial cells which allows for loss of cell polarity, cellular atypia, and local invasion could be studied in this model, as epithelium from various stages in the neoplastic process is readily available for electron microscopy and immunohistochemistry. A morphologic study of keratinization in metaplastic, preneoplastic and neoplastic epithelium could be conducted. The role of inflammatory cells in preneoplasia and neoplasia might be clarified in an in vivo model such as this one. The model may be useful to study the morphogenesis of cartilage tumors or perhaps, the cellular features of spindle cell carcinomas.

The immunologic response to the development of bronchogenic carcinoma could be studied in this model. If specific immunologic markers of respiratory preneoplasia or early neoplasia could be found, and if a similar immunologic response was mounted in humans, a means of early detection of bronchogenic carcinomas might be established.
Because of the practicality of the model, large scale studies could be instituted to study the effects of specific treatments or dietary manipulation on bronchogenic carcinoma. This is one avenue which we will pursue in the future. This model is a sensitive in vivo means of assessing the effect of ascorbic acid in carcinogenesis. If ascorbic acid can, in fact, partially negate oxygen free radical mediated tumor promotion or other stages in carcinogenesis, one might advocate that individuals with a high risk of developing bronchogenic carcinoma (for example asbestos miners who smoke), ingest high daily doses of ascorbic acid, particularly in light of ascorbic acid's non-toxic and inexpensive qualities.
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