EXPERIMENTAL CANCEROUS CHANGES IN THE LUNG
INDUCED BY CHEMICAL CARCINOGENS IN RABBITS*1

(Plates LXIX~LXXI)

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Synopsis

An attempt was made to induce a bronchial carcinoma similar to that of human
in the mucosa of main bronchus of the rabbit by repeated intrabronchial application
of 3-methylcholanthrene through a specially equipped bronchoscope.
1) A strikingly high incidence of squamous metaplasia occurred in the bronchial
mucosa. Eight rabbits (approximately 7%) showed an invasion of atypical squamous
epithelium into the submucosa. Similar changes in the terminal bronchi and/or
alveolar walls were observed in 7 rabbits (approximately 6%). On the other hand,
4 rabbits developed adenomatous proliferation in the submucosa.

These histological features seemed to be indistinguishable from those of carcinom-
atous changes found in human, though no metastases occurred in these rabbits.
2) Combination of the subcutaneous injection of 4-nitroquinoline 1-oxide with
the intrabronchial application of 3-methylcholanthrene was confirmed to be more
effective in inducing the carcinomatous changes on the bronchial mucosa.
3) Applications ranging approximately from 30 to 70 times were found to be most
effective in carcinogenesis, but more than 100 applications caused injury of the
bronchial mucosa which resulted in less incidence of carcinomatous changes.
4) Approximately 60% of the animals developed systemic amyloidosis of rabbits
which had undergone the intrabronchial 3-methylcholanthrene over 50 times.

INTRODUCTION

Experimental lung cancer induced by chemical carcinogens has long been reported
in rats and mice by a number of investigators. However, to our knowledge, only a few
literatures have appeared on the experimental lung cancer in rabbits. Therefore, this
study was undertaken to ascertain whether repetition of direct application of 3-methyl-
cholanthrene*4 on the bronchial mucosa could induce the malignancy in the rabbit.

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*4 The nomenclature of methylcholanthrene has been changed from 20-methyl- to 3-methyl-
cholanthrene in accordance with IUPAC nomenclature rule (A-23.1). The numbering and
structure orientation of cholanthrene are given below:

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H2C\( ^1\)CH\( ^2\)
   \( ^3\)\( ^4\)\( ^5\)\( ^6\)\( ^7\)\( ^8\)\( ^9\)\( ^10\)\( ^11\)\( ^12\)
   \( ^3\)\( ^4\)\( ^5\)\( ^6\)\( ^7\)\( ^8\)\( ^9\)\( ^10\)\( ^11\)\( ^12\)
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The results obtained so far indicated that most of rabbits develop squamous metaplasia on the surface epithelium of the bronchi in the site applied with 3-methylcholanthrene, few developed more outstanding lesions which seemed indistinguishable from those of malignancy.

Moreover some of them were unexpectedly observed to develop systemic amyloidosis accompanying nephrotic syndrome, the details of which were reported elsewhere previously.13)

This paper will concern the results of experimental lung cancer and the implication of its malignancy in the systemic amyloidosis of rabbits caused by repetition of direct application of 3-methylcholanthrene on the bronchial mucosa.

MATERIALS AND METHODS

The animals used throughout the experiments were adult rabbits of both sexes, weighing 3-4 kg, and divided into two groups. One group received the intrabronchial applications of 3-methylcholanthrene alone and the other of 3-methylcholanthrene in combination with subcutaneous injections of 4-nitroquinoline 1-oxide.

For the intrabronchial application, a specially equipped bronchoscope was inserted into the right main bronchus under ether anesthesia. The mucous membrane was swabbed several times through the bronchoscope with pieces of cotton tightly fixed to the tip of the stick dipped in 10% suspension of 3-methylcholanthrene in Tween 60. To protect the rabbit from infection, penicillin, streptomycin, or erythromycin was injected intramuscularly on the same day when 3-methylcholanthrene was given. A solution of 0.5 mg of 4-nitroquinoline 1-oxide dissolved in 0.1 ml of olive oil containing 3% of cholesterol was injected subcutaneously to each rabbit, two or three times a week. Application of the chemical carcinogens was continued unless the rabbits became severely ill or dead. Histological examinations were made of rabbits which happened to die or became severely ill in the course of the experiments.

For the histological examination, each organ was fixed in 10% formaldehyde solution. The thin sections of the main bronchus where 3-methylcholanthrene had been applied, were made for Hematoxylin-Eosin (H-E) staining. Periodic acid-Schiff (PAS) and silver staining were performed when needed. In order to examine the terminal bronchi and alveolar walls carefully, the sections of the lung parenchyma of the side applied were prepared as described above.

Kidneys, spleen, liver, and adrenals were also similarly processed for histological sections which were stained with H-E, PAS, Congo Red, Azan-Mallory, van Gieson, or by thioflavin-T technique for fluorescence. Toluidine Blue was used for metachromasia detection.

RESULTS

In the terminology of histological alterations used herein, "squamous metaplasia" means appearance of squamous epithelium without cornification in bronchial mucosa and/or bronchial glands. If these squamous cells lost their polarity and were atypical in their morphology such as showing pyknosis, dyskeratosis, or mitosis, it was classified as "squamous atypia." There are some alterations called adenomatous proliferation which indicate granulomatous growth involving glandular structure of the bronchus.
and the actively growing epithelial cells being small tubular, acinar, or ductal in appearance.

Criteria for the topographic alterations are based on the structure of the bronchial wall. If the change proceeded to the areas of glands, it is called “that in glands,” and if the alteration underwent further in submucosal tissue, it was called “that in submucosa.” The alterations in “terminal” indicate that they are distributed to the terminal bronchi, distal to the bronchus, especially around the junction of bronchi and alveolar ducts.

**Group 1. Intrabronchial Application of 3-Methylcholanthrene Alone**

This group consisted of a total of 113 rabbits subjected to repeated intrabronchial applications of 3-methylcholanthrene alone on the mucosa of the right main bronchus.

In Table I are presented incidence of the pathological changes found in the mucosa of the right main bronchus and its terminal bronchi, according to which layer the changes appeared among the three layers of the surface epithelium, glands, or submucosa and what type of changes developed. As indicated in Table I, the incidence of squamous metaplasia was approximately 66% (75 rabbits) in the surface epithelium and approximately 48% (54 rabbits) even in the layer involving the glands, whereas it was approximately 33% (37 rabbits) in the terminal bronchi and alveolar walls.

The squamous atypia extending from the surface epithelium to the submucosa was observed in 8 rabbits (approximately 7%) (Photos 1, 2, 5, 6, and 9), while the adenomatous proliferation in 4 rabbits (Photos 3, 7, and 8), 2 of which developed these two pathological changes in some lesions (Photo 4).

These histological features seemed to be quite indistinguishable from those of squamous cell carcinoma or adenocarcinoma found in human bronchial carcinoma. Although the squamous atypia or adenomatous proliferation invaded into the submucosa adjacent to the cartilages, there appeared neither destruction of the cartilages nor metastases to the regional lymph nodes. In 7 rabbits (approximately 6%), growth of the squamous atypia extended into the bronchiolar lumen and/or alveolar walls (Photos 10−12).

<table>
<thead>
<tr>
<th>Pathological findings</th>
<th>Lesions</th>
<th>Surface epithelium</th>
<th>Glands</th>
<th>Submucosa</th>
<th>Terminal bronchi and/or alveolar walls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous metaplasia</td>
<td>75</td>
<td>(66.4±7.7)%</td>
<td>54</td>
<td>(47.8±6.5)%</td>
<td>(32.7±5.4)%</td>
</tr>
<tr>
<td>Squamous atypia</td>
<td>9</td>
<td>(8.0±2.7)%</td>
<td>15</td>
<td>(13.3±3.5)%</td>
<td>(7.1±2.5)%</td>
</tr>
<tr>
<td>Adenomatous proliferation</td>
<td>4</td>
<td>(3.5±1.8)%</td>
<td>8</td>
<td>(6.2±2.3)%</td>
<td></td>
</tr>
</tbody>
</table>

(Error includes statistics only)
Group 2. Combined Intrabronchial Applications of 3-Methylcholanthrene with Subcutaneous Injection of 4-Nitroquinoline 1-Oxide

Table II illustrates incidences of squamous metaplasia in each layer of "surface epithelium," "glands," and "terminal bronchi or alveolar walls" in Group 2. This incidence appeared to be no more than those in the group given intrabronchial application of 3-methylcholanthrene alone. As shown in Table II, however, incidences of squamous atypia in each layer of the bronchus were higher than those in Group 1.

<table>
<thead>
<tr>
<th>Pathological findings</th>
<th>Lesions</th>
<th>Surface epithelium</th>
<th>Glands</th>
<th>Submucosa</th>
<th>Terminal bronchi and/or alveolar walls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous metaplasia</td>
<td>19</td>
<td>(65.5±15.2)%</td>
<td>10</td>
<td>(34.5±11.0)%</td>
<td>(41.4±12.1)%</td>
</tr>
<tr>
<td>Squamous atypia</td>
<td>10</td>
<td>(34.5±11.0)%</td>
<td>10</td>
<td>(34.5±11.0)%</td>
<td>(24.1±9.0)%</td>
</tr>
<tr>
<td>Adenomatous proliferation</td>
<td>2</td>
<td>(6.9±4.8)%</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Relation of the Frequency of the Intrabronchial 3-Methylcholanthrene Applications to Pathological Changes

As indicated in Table III, no correlation was found between the frequency of methylcholanthrene application and carcinomatous changes in the bronchus. Pathological changes invading into the submucosa and histologically indistinguishable from those who had no submucosal invasion were observed in 5 rabbits when the bronchus had been exposed to 51 or more applications of methylcholanthrene. On the other hand, among the 7 rabbits in Group 1, who had received intrabronchial application of methylcholanthrene only, none had such changes.

Table III. Relation of Pathological Findings to Number of Methylcholanthrene Application

<table>
<thead>
<tr>
<th>Pathological findings</th>
<th>Number of application</th>
<th>Under 50 times (No./57)</th>
<th>Over 51 times (No./56)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous metaplasia in terminal bronchi and/or alveolar walls</td>
<td>18</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Squamous atypia in terminal bronchi and/or alveolar walls</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Adenomatous proliferation in submucosa</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Squamous atypia in submucosa</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Squamous atypia in surface epithelium and/or glands</td>
<td>11</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Squamous metaplasia in surface epithelium and/or glands</td>
<td>43</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Systemic amyloidosis</td>
<td>1/23</td>
<td>23/41</td>
<td></td>
</tr>
</tbody>
</table>
of squamous cell carcinoma were found in 8 rabbits, 6 of which had received less than 50 applications of 3-methylcholanthrene over 50~70 days of survival after the initiation of the experiment. The remaining 2 rabbits had received 71 or 146 applications during a period of 105 or 529 days, respectively, until their death.

Four rabbits developed adenomatous proliferation in the submucosa which appeared histologically quite similar to adenocarcinoma. Two of these 4 had received less than 50 applications over 54 and 72 days of survival after the initiation of the experiment and another 2 rabbits, 71 and 80 applications over 105 and 112 days of survival, respectively.

Incidence of squamous metaplasia or squamous atypia in surface epithelium and glands were likely to be higher in the rabbits having had less than 50 applications, while no pathological changes developed in rabbits which had received less than 10 applications over less than 2 weeks of survival. The same holds true for Group 2.

**Incidental Development of Systemic Amyloidosis in Rabbits given Intra-bronchial Applications of 3-Methylcholanthrene**

As seen in Table III, 23 of 41 rabbits (approximately 60%) having had more than 50 applications over more than 3 months of survival after the initiation of the experiment were found to have developed systemic amyloidosis. In contrast, systemic amyloidosis occurred only in one of rabbits which had received less than 50 applications. The kidneys appeared to be markedly swollen grossly and amyloid was found to be deposited in kidneys, spleen, liver, and adrenals. This was confirmed to be amyloid in nature by the histological staining techniques such as H-E, PAS, Congo Red, and thioflavin-T staining for fluorescence. The details of the histological study on the systemic amyloidosis had been previously published.

Table III indicates an interesting correlation between the development of systemic amyloidosis and severity of carcinomatous changes of the bronchial mucosa. Of 24 rabbits developing systemic amyloidosis, 7 (approximately 30%) showed no changes in the bronchial mucosa, 16 squamous metaplasia, and only one invasion of squamous atypia into the submucosa.

**DISCUSSION**

A large bulk of literature on the experimental production of pulmonary tumors has been concerned mainly with that developed in mice and rats for the past 40 years.

The carcinogens used so far are tar, various kinds of carcinogenic hydrocarbons, urethan, isonicotinic acid, radioactive substances or irradiation, and 4-nitroquinoline 1-oxide. The manner in which the carcinogens are administered includes intratracheal, intranasal, oral, aspiratory application, intraperitoneal, intravenous, subcutaneous injection, direct implantation into the lungs, and painting over the skin.

Murphy et al. first reported an increase in the incidence of spontaneous pulmonary tumor in mice painted over the skin with tar. Shortly later, various kinds of carcinogenic hydrocarbons were proved to be capable of inducing malignancy when applied on the skin. Furthermore, Mori et al. recently succeeded in inducing lung cancer in mice and rats by injecting 4-nitroquinoline 1-oxide subcutaneously.
However, direct application of the carcinogenic hydrocarbons into the lungs seemed to result in lower incidence of carcinogenesis. This may be attributed to the prompt removal of the carcinogens from the lungs.\textsuperscript{18,42} The prolonged stay of the carcinogens applied locally seems to be an essential prerequisite for the induction of carcinogenesis. For this reason, many new techniques have been devised to keep the carcinogens in the lungs as long as possible, such as intratracheal intubation of carcinogenic hydrocarbon mixed with powder of black ink,\textsuperscript{41,42} implantation of pellets containing radioactive substances,\textsuperscript{19,20} carcinogen-soaked thread into the lungs,\textsuperscript{2,18} or application of the carcinogen into the area of the lungs infarcted artificially.\textsuperscript{43} These methods have proved much more efficient in the production of lung cancer.

Although it had long been considered very difficult to induce the bronchial carcinoma experimentally in rabbits, our challenge was made to induce the experimental bronchial carcinoma in rabbits indistinguishable from that of the human by repeated application of 3-methylcholanthrene on the bronchial mucosa through a specially equipped bronchoscope. Repetition of the carcinogen application was presumed to be crucial for their prolonged stay on the main bronchus. Against this presumption, the results obtained showed no positive correlation between the frequency of the intrabronchial applications and severity of the carcinomatous changes developed.

In this series of the experiment, majority of the rabbits developing striking carcinomatous changes on the bronchial mucosa had received approximately 50 applications. However, many of the rabbits receiving more than 100 applications showed no carcinomatous changes and the right main bronchial mucosa of these rabbits were apt to be desquamated and the cartilages beneath the submucosa exposed.

The appearance of the carcinomatous changes observed on the main bronchial wall was characterized by the invasion of squamous atypia into the submucosa or of adenomatous proliferation. These changes were limited only to the submucosa, never extending into the cartilages, resulting in destruction. The cartilages beneath the submucosa were likely to hinder the induction of carcinogenesis.

In a few rabbits, growth of squamous atypia was encountered in the terminal bronchi or alveolar walls of the lung on the bronchus to which 3-methylcholanthrene had been applied. Thus, the induction of carcinogenesis would be expected to occur in the pulmonary parenchyma where hinderance of carcinogenesis by cartilages does not exist.

Combination of subcutaneous injection of 4-nitroquinoline 1-oxide with intrabronchial application of 3-methylcholanthrene was shown to result in a higher incidence of squamous atypia in each site of the bronchus. Combination of a variety of chemical carcinogens would be expected to be more effective in inducing carcinoma.

Although rabbits developing carcinomatous changes were found to die easily of pneumonia or other infections, further observation is needed to ascertain what type of carcinomatous changes would occur in rabbits surviving over a prolonged period of time.

Approximately 60% of rabbits which had received the intrabronchial application of 3-methylcholanthrene on more than 50 occasions happened to develop systemic amyloidosis. The majority of these rabbits showed only minor pathological changes of the bronchial walls. In contrast, most of the rabbits developing carcinomatous changes did not develop systemic amyloidosis.
The mechanism leading to the systemic amyloidosis remains obscure at the present time, though a hypothesis could be made that 3-methylcholanthrene applied intrabronchially might be metabolized to act as an amyloidogen to the endothelial cells.

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EXPLANATION OF PLATES LXIX-LXXI

Photo 1. Squamous atypia in the submucosa. Medium sized cell nests were observed with irregular distribution in submucosal tissue. Rabbit No. H-71 with 39 intrabronchial applications of 3-methylcholanthrene over 54 days of survival. H-E. ×80

Photo 2. Squamous atypia in the submucosa. Elevated mucosal lesions were characterized by solid sheets of atypical squamous epithelium. This pattern was suggestive of atypia of the bronchial glands, since a few small lumens in the middle of the sheets were scattered. These lesions were situated in the submucosal layer. Rabbit No. H-62 with 45 intrabronchial applications of 3-methylcholanthrene over 60 days of survival.

Photo 3. Adenomatous proliferation of the bronchial glands. H-E. ×80. Lesions of atypical proliferation of glands extended into the submucosal tissue and muscle layer between the cartilages. Rabbit No. H-54 with 46 intrabronchial applications of 3-methylcholanthrene over 72 days of survival. H-E. ×80

Photo 4. Combined lesion of squamous atypia with adenomatous proliferation. This picture shows atypical squamous metaplasia in the right upper field and adenomatous proliferation in the left upper field. Atypical glandular pattern seemed to extend to the outer aspect of the bronchial cartilage. Rabbit No. H-15 with 71 intrabronchial applications of 3-methylcholanthrene over 105 days of survival. H-E. ×80

Photo 5. A strikingly magnified view of squamous atypia shown in Photo 1. The lesion was characterized by small squamous cell cords infiltrating into stroma. H-E. ×400

Photo 6. A highly magnified view of atypical squamous metaplasia shown in Photo 2. Solid nests of disorganized arrangement and variety of cells in shape and size were noted. H-E. ×200

Photo 7. A highly magnified view of adenomatous proliferation shown in Photo 3. Monolayer or multilayers of glandular structure were lined by ciliated surface of mucous and/or serous epithelial cells. H-E. ×200

Photo 8. A highly magnified view of adenomatous proliferation shown in Photo 4. H-E. ×200

Photo 9. A highly magnified view of a atypical squamous metaplasia shown in Photo 4. H-E. ×200

Photo 10. Atypical squamous metaplasia in the terminal bronchus. A cross section of a terminal bronchus was characterized by lining epithelium of squamous atypia. In the lumen, polymorphonuclear leucocytes, histiocytes and mucous material excreted were observed. Rabbit No. H-22 with 40 intrabronchial applications of 3-methylcholanthrene over 56 days of survival. H-E. ×400

Photo 11. Atypical squamous metaplasia in the terminal bronchi. Terminal bronchi were surrounded by numerous inflammatory exudates and necrotic tissue. From the same rabbit as shown in Photo 10. H-E. ×100

Photo 12. A highly magnified view of atypical squamous metaplasia in the terminal bronchi shown in Photo 11. The surface epithelium was remarkably thickened and variable in shape and size. Mitoses were abundant. H-E. ×400

H-E=Hematoxylin and Eosin stain.