Induction of Lung and Exocrine Pancreas Tumors in F344 Rats by Tobacco-specific and Areca-derived N-Nitrosamines\textsuperscript{1,2}

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ABSTRACT

The tobacco-specific N-nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanal (NNAL), as well as the Areca-derived N-nitrosoguacoline (NG) were assayed for carcinogenicity in male F344 rats by lifetime administration in the drinking water. Groups of 30 to 80 rats were treated with 0.5 ppm, 1.0 ppm, or 5.0 ppm of NNK; 5.0 ppm of NNAL, 20 ppm of NG, a mixture of 20 ppm of NG and 1 ppm of NNK, and water only in the control group. The approximate total doses of the nitrosamines (mmol/kg of body weight) in these groups were: NNK, 0.073, 0.17, and 0.68; NNAL, 0.69; NG, 4.1; and NG and NNK, 4.1 and 0.17. As in previous assays in which NNK was tested by s.c. injection, the lung was its principle target organ. Lung tumor incidences in the 0.5-, 1.0-, and 5.0-ppm groups were nine of 80, 20 of 80, and 27 of 30 compared to six of 80 in the control rats. This trend was significant, \( P < 0.005 \). Significant incidences of nasal cavity and liver tumors were observed only in the rats treated with 5.0 ppm of NNK. In contrast to the results of the s.c. bioassays of NNK, tumors of the exocrine pancreas were observed in five of 80 and nine of 80 rats treated with 0.5 and 1.0 ppm. This trend was significant, \( P < 0.025 \). This is the first example of pancreatic tumor induction by a constituent of tobacco smoke. It is also the first finding of duct-like carcinomas in the rat pancreas, including one tumor containing epidermoid, keratin-generating tissue. NNAL, the major metabolite of NNK, induced lung tumors in 26 of 30 rats and pancreatic tumors in eight of 30 rats. It appears to be the proximate pancreatic carcinogen of NNK. NG induced pancreatic tumors in four of 30 rats, \( P < 0.05 \). This finding requires confirmation. The mixture of NG and NNK induced lung tumors in eleven of 30 rats. There were no apparent synergistic interactions of NG and NNK. The observation of benign and malignant tumors of the lung and pancreas of rats treated with the tobacco-specific nitrosamines NNK and NNAL is discussed in respect to the causal association between cigarette smoking and cancer of the lung and pancreas.

INTRODUCTION

Epidemiologically, cigarette smoking is causally linked with cancer of the lung, larynx, oral cavity, esophagus, pancreas, renal pelvis, and urinary bladder and is also associated with cancer of the nasal cavity and cervix. Smoking of cigars and pipes is causally related to cancer of the respiratory tract, oral cavity, and esophagus, although, in the case of lung cancer, not to the same extent as cigarette smoking (1–3). Chewing of tobacco, and especially the oral use of snuff, is associated with cancer of the oral cavity and, possibly, with cancer of the nasal cavity, pancreas, kidney, and bladder (4–8). The habit of chewing tobacco-containing betel quid is known to lead to cancer of the mouth and of the esophagus (4).

The TSNA\textsuperscript{1} (Fig. 1) are the most abundant, strong carcinogens in chewing tobacco, snuff, tobacco-containing betel quid, and tobacco smoke (4, 6, 9, 10). They are formed by N-nitrosation of nicotine (1 to 2% of the tobacco) during processing and storage. In cigarette smoke, 26 to 37% of NNK and 40 to 46% of NNN originate from tobacco by direct transfer; the remainder is pyrosynthesized during smoking (11, 12). The N-nitrosamines generated as a result of the N-nitrosation of arecoline, the major alkaloid in betel quid, are NG, NGC, MNPN, and MNPA (Fig. 2; Ref. 13).

The levels of NNN and NNK in chewing tobacco (1 to 8.5 ppm), snuff (3 to 100 ppm), and cigarette smoke (0.2 to 4 µg/ cigarette) are generally at least two orders of magnitude higher than the concentrations of carcinogenic N-nitrosamines in other consumer products or respiratory environments (10, 14, 15). Mixtures of betel quid and tobacco also contain NNN (0.025 to 0.1 ppm), NNK, and NG (up to 0.014 ppm) (16). NNK and NNN induce benign and malignant tumors in the nasal cavity, oral cavity, esophagus, lung, and/or liver of mice, rats, and hamsters (10, 17), while NNAL causes lung tumors in mice (17). NNK is considered to be more carcinogenic in F344 rats than N-nitrosodimethylamine (18, 19). Lijinsky and Taylor reported that NG, the major nitrosation product of arecoline, was not carcinogenic when given to rats in drinking water (20).

NNK, the most carcinogenic compound in the TSNA group, had not been previously tested by administration in the drinking water. Its presence in the saliva of tobacco chewers, tobacco smokers, and chewers of tobacco-containing betel quid (16, 21–23) warranted testing by p.o. application. NNAL, the major metabolite of NNK (24–26), had not been previously tested for carcinogenicity in rats. In this lifetime bioassay, male F344 rats were given 0.5, 1.0, or 5.0 ppm of NNK or 5.0 ppm of NNAL in the drinking water. We also assayed NG (20 ppm) and a mixture of NNN (1.0 ppm) and NG (20 ppm) in a ratio comparable to that found in the saliva of chewers of tobacco-containing betel quid (13, 22).

MATERIALS AND METHODS

Chemicals. NNK, NNAL, and NG were synthesized according to earlier published methods. They were greater than 99% pure according to gas chromatography and high-performance liquid chromatography analyses (26–28). The solutions of the N-nitrosamines were newly prepared every 2 wk and were stored in amber bottles in a cold room prior to administration.

Bioassays for Carcinogenicity. Male F344 rats, 6 wk old, were obtained from Charles River Breeding Laboratories, Kingston, NY. When the rats were 8 wk old, the bioassay was started. The rats were housed in groups of 3 in solid-bottomed polycarbonate cages with hardwood bedding under standard conditions [20 ± 2°C (SD); 50 ± 10% relative humidity; 12-h light and dark cycle]. NIH-07 diet and tap water with or without N-nitrosamines were given ad libitum. The 500-ml amber bottles were filled with the drinking water preparations every seventh day, and the fluid consumption was recorded.

The bioassay consisted of the following groups: I, 0.5 ppm of NNK, 80 rats; II, 1.0 ppm of NNK, 80 rats; III, 5.0 ppm of NNK, 30 rats; IV, 5.0 ppm of NNN, 30 rats; V, 20 ppm of NG, 30 rats; VI, 20 ppm of NG plus 1.0 ppm of NNK, 30 rats; and VII, negative control group (water only), 80 rats.
addition, the crowding and "piling up" process, never seen in the normal pancreas, was always present in these acinar carcinomas. Mitoses were frequently found, and they appeared hyperchromatic and sometimes multipolar.

In some larger well-differentiated acinar adenocarcinomas, the acinar pattern was broken by a tendency to form papillary structures. It is noteworthy that the few acinar carcinomas described in the human pancreas had similar patterns (32).

The second type of carcinomas of exocrine origin was formed mostly of duct-like structures of various dimensions and shapes, combined with "solid" areas as well as papillary or trabecular aspects (Figs. 3 to 8). Mucous material was present in and outside the tubular lumina. One of the rats had a pancreatic tumor with intermixed areas of ductal, endocrine, and epidermoid (keratinizing) tissue (Figs. 6 to 8).

Although the number of rats with exocrine tumors of the pancreas in Group I (Table 2) was not significantly different from that in the negative control (Group VII), Bartholomew's test showed a significant positive trend between the control Group VII, Group I (0.5 ppm of NNK), and Group II (1.0 ppm of NNK; P < 0.025).

As in earlier bioassays in which we applied it by s.c. injection (19, 20), NNK proved to be a powerful lung carcinogen in rats also when applied p.o. The difference in lung tumor incidence between Group I (0.5 ppm of NNK) and the control group was not statistically significant; yet Bartholomew's test (29) showed a highly significant trend for lung tumors across exposure levels (Groups VII, I, II, and III; P < 0.005). NNAL was also a potent lung carcinogen, with activity similar to that of NNK. The morphology of the adenomas and adenocarcinomas in the lung did not differ markedly from that observed after s.c. injections of NNK (18, 19). Epidermoid metaplastic foci occurred in large areas of these tumors and generated squamous carcinomas in greater number than after s.c. injection of NNK.

Surprisingly, the incidence of tumors of the nasal cavity was lower than that observed upon s.c. injection of NNK (18, 19). The incidence of the nasal cavity tumors was significant only in the group treated with the highest dose of NNK (Group III). Liver tumor incidence in this bioassay appears to be similar to that observed in previous assays of NNK (18, 19). Liver tumors were induced in 12 of 30 rats in Group III (total dose, 0.68 mmol/kg) and in 11 of 80 rats receiving 0.17 mmol/kg. In the negative control group, 6 of 80 rats developed liver tumors. Upon s.c. injection, a dose of 0.33 mmol/kg induced liver tumors in 10 of 27 rats (19).
TABLE 1 Uptake of N-nitrosamines, survival time, water consumption, and body weights of male F344 rats

<table>
<thead>
<tr>
<th>Group</th>
<th>N-nitrosamine</th>
<th>Dose, mg/rat</th>
<th>Dose, mmol/kg</th>
<th>Av. survival (wk)</th>
<th>No. of rats</th>
<th>Body wt of rats at wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NG, 0.5 ppm</td>
<td>6.9 ± 1.8</td>
<td>0.073</td>
<td>103.3 ± 14.3</td>
<td>80</td>
<td>389.9 ± 22.2</td>
</tr>
<tr>
<td>II</td>
<td>NNK, 1.0 ppm</td>
<td>15.6 ± 3.3</td>
<td>0.17</td>
<td>101.5 ± 11.9</td>
<td>80</td>
<td>386.1 ± 18.3</td>
</tr>
<tr>
<td>III</td>
<td>NNK, 5.0 ppm</td>
<td>63.5 ± 11.6</td>
<td>0.68</td>
<td>90.1 ± 11.8</td>
<td>30</td>
<td>390.7 ± 20.0</td>
</tr>
<tr>
<td>IV</td>
<td>NNAL, 5.0 ppm</td>
<td>66.6 ± 14.5</td>
<td>0.69</td>
<td>93.0 ± 16.2</td>
<td>30</td>
<td>386.9 ± 21.3</td>
</tr>
<tr>
<td>V</td>
<td>NG, 20 ppm</td>
<td>315 ± 68.6</td>
<td>4.1</td>
<td>105.9 ± 17.2</td>
<td>30</td>
<td>381.1 ± 20.5</td>
</tr>
<tr>
<td>VI</td>
<td>NG + NNK, 20 ppm</td>
<td>313 ± 60.6</td>
<td>4.1 ± 0.17</td>
<td>109 ± 13.7</td>
<td>80</td>
<td>385.0 ± 20.4</td>
</tr>
<tr>
<td>VII</td>
<td>water only</td>
<td>128</td>
<td>4.1 ± 0.17</td>
<td>109 ± 13.7</td>
<td>128</td>
<td>450.3 ± 26.5</td>
</tr>
</tbody>
</table>

Table 2 Tumor incidence in male Fischer rats upon administration of N-nitrosamines in drinking water

<table>
<thead>
<tr>
<th>Group</th>
<th>N-nitrosamine</th>
<th>No. of male F344 rats</th>
<th>No. of rats with testicular Leydig tumors</th>
<th>No. of rats with liver tumors</th>
<th>No. of rats with lung tumors</th>
<th>No. of rats with exocrine pancreas tumors</th>
<th>No. of rats with nasal cavity tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NNK, 0.5 ppm</td>
<td>80</td>
<td>2</td>
<td>5</td>
<td>5</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>NNK, 1.0 ppm</td>
<td>80</td>
<td>9</td>
<td>10</td>
<td>12</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>III</td>
<td>NNK, 5.0 ppm</td>
<td>30</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>IV</td>
<td>NNAL, 5.0 ppm</td>
<td>30</td>
<td>3</td>
<td>12</td>
<td>12</td>
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<td>6</td>
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<tr>
<td>V</td>
<td>NG, 20 ppm</td>
<td>30</td>
<td>5</td>
<td>9</td>
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<td>6</td>
</tr>
<tr>
<td>VI</td>
<td>NG + NNK, 20 ppm</td>
<td>10</td>
<td>10</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>VII</td>
<td>water only</td>
<td>80</td>
<td>24</td>
<td>15</td>
<td>20</td>
<td>10</td>
<td>51</td>
</tr>
</tbody>
</table>

For each of these tumors were ductal adenocarcinomas.

The incidence rates of the testicular Leydig tumors were significantly less than those in the control (Group VII).

The number of rats with testicular Leydig tumors was significantly less than controls in all N-nitrosamine groups except Group VI.

DISCUSSION

This bioassay resulted in a number of important observations. First, NNK and NNAL, when given in the drinking water, induced large adenomas and adenocarcinomas of the exocrine pancreas. Although exocrine pancreatic tumors have been experimentally induced with synthetic agents (33-37), the present...
TOBACCO-RELATED TUMORS OF THE LUNG AND PANCREAS IN RATS

results are the first examples of induction of pancreas tumors in laboratory animals with an agent present in tobacco and tobacco smoke. These data are supported by the positive dose-dependent trend in Groups VII, I, and II. The low and insignificant yield of pancreas tumors observed in Group III (5.0 ppm of NNK) may be due to the high incidence of tumors of the lung, nasal cavity, and liver which markedly shortened survival (90.1 ± 11.8 wk) by comparison to the control rats (108.0 ± 11.5 wk). A comparison of the dates of appearance of lung tumors in Group III (5.0 ppm of NNK) and Group II (1.0 ppm of NNK) by the Mann-Whitney U test (30) showed a significant delay of onset of tumors in the lower dose group (P < 0.001).

The highest incidence of benign and malignant pancreas tumors (8 of 30 rats) occurred in the rats receiving 5.0 ppm of NNAL (Group IV), indicating that this enzymatic reduction product of NNK is likely the proximate pancreas carcinogen. Upon i.v. injection in male F344 rats, NNK is rapidly converted to NNAL. The biological half-life of NNK is 0.4 h compared to 2.9 h for NNAL (24). DNA binding studies have shown that NNAL can be reconverted to NNK, yielding the same DNA adducts as those observed upon in vivo administration of NNK.5 We are studying the role of NNAL in the induction of pancreas tumors by comparing the degree and persistence of DNA adduct formation in the pancreas of rats treated with NNK and NNAL.

In terms of histogenesis, the acinar carcinomas of the pancreas cannot be morphologically traced to the progression towards malignancy of an (acinar) adenoma, although this possibility is not excluded. However, the exocrine nonacinar car-

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TOBACCO-RELATED TUMORS OF THE LUNG AND PANCREAS IN RATS

Fig. 6. Low power of a mixed islet and duct pancreas carcinoma nodule. Extensive keratinizing squamous metaplasia. (Rat treated with NNAL.)

Fig. 7. Enlarged area of squamous keratinizing areas of the tumor of Fig. 6.

Fig. 8. High-power view of the same (Fig. 6) tumor. Tumorous duct with squamous metaplasia in its wall, protruding into the lumen of the duct.

cinomas most probably have their origin in the small ducts or ductules, as Pour et al. have described for hamsters (33). The cells of the intercalated ductules (Boll ductules), including the centroacinar cells, seem to have regenerative histogenetic capacity similar to the basal (reserve) cells of various mucosal epithelia. The histological images suggest that they can differentiate along various directions forming acinar, ductal, or even Langerhans tissue. Ductular-centroacinar cells seem to be the cells primarily affected by the carcinogen. Regarding the pancreatic tumor containing metaplastic squamous tissue and keratin (Figs. 6 to 8), this is, to our knowledge, the first observation of an experimental carcinoma of the pancreas with epidermoid component. This strengthens the assumption that ductular cells can function as multipotential stem-cells and be the generator of pancreas cancers (36).

One goal of this study was to see whether NG, the major N-nitrosamine in betel quid, affects the tumorigenic potency of NNK, which is present in tobacco-containing betel quid and in the saliva of chewers of these quids (16, 22). Betel quid chewing has long been associated with oral cancer in India and many Asian countries. In fact, among the more than 100 listings established in population-based registries around the world, India has the highest rate of oral cancer. So far N-nitrosamines are the only known carcinogens in betel quid (4). It was, therefore, important to explore the possible synergistic effect between NG and NNK. When NNK and NG were concomitantly given in the drinking water in a ratio of 1 to 20 ppm (Group VI), tumor yields observed in these rats were not significantly different from those in the rats in Group II (1.0 ppm of NNK). This applied to tumors of the lung, pancreas, nasal cavity, and liver. When NG was applied alone (Group V), the occurrence of acinar tumors of the exocrine pancreas (4 of
provides additional carcinogen exposure (9). This study generation is the possibility of endogenous formation of NNK from They are based on several assumptions. One important consid
exposed to about 250 mg of NNK, or approximately 3.6 mg/l
smoker of a United States nonfilter cigarette (425 ng of NNK/
nicotine upon smoke inhalation or during chewing which likely
lung induced by the lowest dose of NNK (0.073 mmol/kg) were
though the incidence rates of tumors of the pancreas and of the
at a total dose of only 15.6 ± 3.3 mg of NNK per rat (Group

ACKNOWLEDGMENTS

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REFERENCES

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