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A Study of Tobacco Carcinogenesis : Effect of the Fat Content of the Diet on the Carcinogenic Activity of 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone in F344 Rats[1, 2]

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ABSTRACT

3 Epidemiological studies indicate that the risk of cigarette smokers for cancer of the lung and of the pancreas is influenced by the fat content of the daily diet. In a long-term bioassay (24 months), we gave F344 rats 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a tobacco-specific and strongly carcinogenic *N*-nitrosamine, as a 2 ppm solution in the drinking water. One group of rats was given a high-corn oil diet (23.5%), and the second group received a low-corn oil diet (5.0%). The animals on the high-corn oil diet and NNK (NNK-HF), and the control animals on the same diet but on tap water (HF) had significantly higher body weights and shorter life spans than the rats on a low-corn oil diet and NNK (NNK-LF) and the corresponding control rats receiving the low-corn oil diet and tap water (LF). Eighteen months into the bioassay, 16 of 60 rats in the NNK-HF group had developed lung tumors averaging 6.8 mm², while 3 of 60 rats in the NNK-LF group had tumors averaging 2.5 mm². At the termination of the experiment after 24 months, the numbers of rats with lung tumors in the NNK-HF and NNK-LF groups did not significantly differ from each other, nor was there a difference in the size of the lung tumors. The effect of dietary fat on the pancreas tumor incidence was more pronounced. After 18 months, 11 of 60 rats treated with NNK-HF but only one of 60 rats treated with NNK-LF had developed pancreas tumors. At the termination of the study, 28 NNK-HF-treated rats had pancreas tumors (17.5 ± 13.5 mm) compared to 19 NNK-LF-treated rats (9.6 ± 11.7 mm²). After 24 months 6 of 20 rats in each of the control groups (HF and LF) had developed pancreas tumors. In fact, there was an increasing trend of development of pancreas tumors in these control rats with aging regardless of dietary fat variance. However, in view of the observed tumor acceleration and enhancement this study points to the importance of evaluating both exposure to tobacco carcinogens and dietary fat intake as risk factors for tobacco users.

INTRODUCTION

Epidemiological studies indicate that the fat content of the daily diet plays an important role in the risk for cancer of the lung and cancer of the pancreas (1–6). Although the effect of dietary fat has been studied in a number of bioassays with carcinogens (7–9), this aspect had not as yet been explored in tobacco carcinogenesis. In this communication we report observations on the influence of a 23.5% corn oil diet *versus* a 5% corn oil diet on the carcinogenic activity of NNK[4] in the F344 rat. NNK and its enzymatic reduction product, NNAL, are nicotine-derived *N*-nitrosamines that occur in smokeless tobacco (0.1–20 μg/g) and in tobacco smoke (0.05–0.5 μg/cigarette). In rats these agents induce primarily adenocarcinoma of the lung and tumors of the exocrine pancreas (10–14). In this lifetime bioassay, we placed male F344 rats on a high-fat diet (23.5% corn oil) and a low-fat diet (5.0% corn oil), while rats in both groups received 2 ppm NNK in the drinking water. On the basis of a previous study in which NNK was given to F344 rats in the drinking water at concentrations of 0.5, 1.0, and 5.0 ppm (11), we calculated that 2.0 ppm would be the most appropriate dose for the observation of modulating effects on the tumor response in the lung and pancreas. In the negative control group, rats were maintained on high- and low-corn oil diets but were given only tap water.

MATERIALS AND METHODS

Chemicals. NNK was synthesized according to an earlier published method. Its purity was greater than 99.5% as verified by gas chromatography and high-performance liquid chromatography (15). The 2 ppm NNK-containing drinking water was prepared every 2 weeks and was stored in amber bottles in a cold room prior to use.

Diets. Beginning at the age of 8 weeks and throughout their lifetimes the male F344 rats (Charles River Breeding Laboratories, Kingston, NY) were maintained on a high-fat diet (23.5% corn oil) or on a low-fat diet (5.0% corn oil). The isocaloric diets were formulated on the basis of the standard reference diet of the American Institute of Nutrition (Ref. 16; Table 1). Corn oil contains about 10% palmitic acid, 31% oleic acid, and 56% linoleic acid. The diets were prepared weekly under standardized conditions as reported by Reddy *et al.* (17).

Bioassays. 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]. The high- and low-corn-oil diets and tap water with or without NNK were given *ad libitum*. The NNK-containing water was offered throughout the lifetimes of the rats beginning at 8 weeks of age. The 500-ml amber bottles were filled with the aqueous solution of NNK every seventh day, and the fluid consumption was recorded. The animals were weighed weekly during the first month and then every 4 weeks until termination of the assay.

The bioassay consisted of the following groups: I, 23.5% corn oil diet, 2 ppm of NNK, 60 rats (NNK-HF); II, 5.0% corn oil diet, 2 ppm NNK, 60 rats (NNK-LF); III, 23.5% corn oil diet, tap water, 20 rats (HF); IV, 5.0% corn oil diet, tap water, 20 rats (LF). Groups III and IV were negative controls.

The animals were observed until moribund or until the scheduled termination of the experiment (when 90% of the rats in the longest surviving group had died) and were killed by CO_2 inhalation. Complete autopsies were performed. Histology slides were prepared for all gross lesions in the lung, liver, spleen, kidney, adrenals, pancreas, esophagus, larynx, and trachea. The sizes of the small lung and pancreas tumors were determined with a Leitz-Möller micrometer on slides according to the method of Longnecker *et al.* (18). Those larger than 1 cm in diameter were measured with a millimetric ruler. The pancreatic neoplasms were catalogued according to the method of Longnecker and Millar (19), with minor modifications. To search for nasal cavity tumors, the head was decalcified, after removing the skin, mandibula, and muscles. The decalcified head was divided into 2 parts through a retroorbital section. The anterior part, containing the nasal cavity, was further divided into 3 fragments by 2 equidistant, frontal cuts. All 3 pieces were embedded in paraffin and processed for microscopic examinations.

Statistical evaluations were completed with the 2-sample *t* test and χ^2 test.

RESULTS

Table 2 presents the average weights of the rats in the four groups at various time intervals, total average consumption of water, uptake

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[4] The abbreviations used are: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; HF, high-fat diet; LF, low-fat diet.

Table 1 *Percentage of components in experimental diets (16)*

Diet ingredients	Corn oil diets	
	Low-fat[a] (5%)	High-fat[b] (23.5%)
Casein	20.0	23.50
D,L-Methionine	0.3	0.35
Cornstarch	52.0	32.90
Dextrose	13.0	8.30
Alphacel	5.0	5.90
Corn oil	5.0	23.52
Mineral mix, AIN	3.5	4.11
Vitamin mix, AIN revised	1.0	1.18
Choline bitartrate	0.2	0.24

[a] This diet was formulated on the basis of the American Institute of Nutrition (AIN) standard reference diet with the modification of varying sources of carbohydrate.

[b] Additional corn oil was added at the expense of starch and dextrose. The composition of the high-fat diet was adjusted so that all animals in various dietary groups would consume approximately the same levels of protein, vitamins, minerals, fiber, and calories (17).

of NNK with the drinking water (NNK-HF and NNK-LF), as well as average survival and week of termination. As to be expected, the average body weights of the animals in the NNK-HF and HF groups were at all times significantly higher ($P < 0.01$) than those in the NNK-LF-treated and corresponding control group, LF. Comparing the weights of the NNK-HF-treated animals and those of their controls, there was no significant difference until week 78; subsequently, the rats in the HF control group lost weight more quickly, as is also reflected in the somewhat shorter survival. Throughout the bioassay the weights of the rats in the NNK-LF and LF groups were about the same. Survival of the rats on the high-fat diet (NNK-HF, 78.5 ± 15.5 weeks; HF, 75.3 ± 13.0 weeks) was significantly shorter ($P < 0.05$) than that of animals on the low-fat diet (NNK-LF, 86.9 ± 13.4 weeks; LF, 90.0 ± 12.6 weeks). The somewhat lesser overall water consumption of the rats in the HF control group is probably related to the fact that these animals appeared moribund earlier. The water consumption charts show that in the experimental groups the rats on the high-fat diet (NNK-HF) consumed more water during their lifetimes than did their counterparts on the low-fat diet (NNK-LF); however, because of the shorter survival of rats in the NNK-HF group compared to the NNK-LF group (78.7 ± 15.1 *versus* 85.5 ± 13.4 weeks), the overall water consumption and thus the exposure to the carcinogenic NNK were similar.

The total number of rats with NNK-related tumors in the NNK-HF group (46 of 60) was insignificantly higher than that in the NNK-LF group (40 of 60; $P > 0.05$). On the basis of many bioassays with NNK in rats, we regard the increase of tumors of the lung, pancreas, nasal mucosa, and liver as NNK-related neoplasms (10–13). Table 3 shows that the rats treated with NNK-HF develop lung tumors earlier than those in the NNK-LF group. After 18 months, 16 of the rats that had died in the NNK-HF group had developed lung tumors averaging 6.8 mm^2, while only 3 of 60 rats in the NNK-LF group had lung tumors with an average size of 2.5 mm^2. However, at the termination of the bioassay (97 and 102 weeks), the numbers of rats with lung tumors in the two groups were similar (30 *versus* 27), as were the tumor sizes (19.6 *versus* 17.8 mm^2). These lung tumors were adenoma or adenocarcinoma, except for an adenosquamous carcinoma in one rat in each group. The detailed morphology of these tumors was described earlier (11, 20, 21). The incidence rates of tumors in the nasal cavity (5 *versus* 4) and the liver (5 *versus* 2) did not differ among the rats in the NNK-HF and NNK-LF groups (Table 3). F344 rats in long-term experiments after 18 months develop heavy infestations of the nasal cavity by an *Aspergillus* fungus (probably *Aspergillus flavus*). In this experiment, the rats with tumors in the nasal cavity had developed them at the site of contact between the mucosa and fungus ball. Unlike the tumors induced by tobacco-specific *N*-nitrosamines, which are ethesioneuroepitheliomas (21), those induced by contact with *Aspergillus* are squamous cell tumors (papilloma or carcinoma).

Table 4 summarizes the occurrence of tumors of the pancreas in the four groups. NNK and its metabolic reduction product NNAL are the only environmental agents known to induce tumors in the pancreas of a laboratory animal (11). The carcinogenic potency of NNK in the pancreas of rats was clearly increased when the animals had been fed a high-corn oil diet (NNK-HF), as can be seen by comparing the numbers of rats with tumors with those in NNK-treated rats on a low-corn oil diet (NNK-LF) (28 of 60 *versus* 19 of 60). In the NNK-HF group, 18 rats had benign and malignant tumors of the exocrine pancreas; in addition 10 islet cell tumors were seen. In the NNK-LF group, the corresponding numbers were 14 and 5. The average size of the exocrine pancreas tumors in the rats in the NNK-HF group was 17.5 ± 13.5 mm^2, compared to 9.6 ± 11.1 mm^2 in rats in the NNK-LF group. The high-fat diet also had an accelerating effect on pancreas tumor development in the NNK-treated rats in that the tumors appeared earlier. After 18 months, 11 of the killed rats had developed pancreas tumors in the NNK-HF group compared to one of 60 rats in the NNK-LF group; after 24 months a total of 28 rats with pancreas tumors had been observed in the NNK-HF group and 19 in the NNK-LF group. It should be mentioned that one of the tumors in the NNK-HF group was the largest pancreatic tumor (acinar adenocarcinoma) ever reported in a rat (50 g; 100 mm long; 3000-mm^2 cut surface at the longest diameter). To allow an overall calculation of the average tumor size, this outlier was counted as being no larger than the second largest pancreas tumor in a rat in the same group (45.5 mm^2). The electron microscopic morphology of this tumor showed a well-differentiated acinar pancreatic carcinoma. Details on the morphology of the pancreatic tumor will be published separately.

DISCUSSION

Cigarette smoking is causally associated with an increased risk for cancer of the lung and pancreas (22, 23). Epidemiological studies suggest that, compared to cigarette smokers on a low-fat diet, cigarette smokers with a high fat intake face an increased risk for both of these types of cancer (1–6). NNK is a major lung carcinogen in tobacco smoke. NNK and its enzymatic reduction product, NNAL, are the only two smoke constituents known to induce pancreas tumors in laboratory animals (10–13). The rats on the high-corn oil diet developed lung and pancreas tumors earlier and more rapidly; moreover, these tumors were of larger size. We regard the size of the tumors to be as important as the total number of tumors, because the larger size indicates an early occurrence and/or accelerated rate of growth.

Interestingly, 6 of 20 rats in control groups HF and LF developed tumors of the pancreas, and the majority of these tumors arose between the 18th and 24th months of the experiment regardless of the variance of dietary fat. This is in line with observations in two unrelated bioassays recently conducted at our institute, suggesting that the percentage of spontaneous exocrine pancreatic tumors in F344 rats is larger than is reported in the literature (19).

In an earlier bioassay, male F344 rats were given 1 and 5 ppm of NNK in the drinking water; however, the animals were on a NIH-07 standard diet (4.5% crude fat; Ref. 16). Since the NNK concentration applied in the drinking water in the current assay was 2 ppm, it is difficult to compare the results of the two studies. Nevertheless, the tumor yields were in the expected ranges, except that the yields of acinar tumors of the pancreas in the NNK-HF group (2 ppm NNK) were significantly above those in the rats on an NIH-07 standard diet upon administration of 1 ppm NNK ($P < 0.01$) and even higher than in those given NIH-07 and 5 ppm NNK ($P < 0.025$).

Table 2 *Uptake of NNK, survival time, water consumption, and body weights of male F344 rats*[a, b]

Week of experiment	Group I NNK-HF[c] 60 rats	Group II NNK-LF[c] 60 rats	Group III HF[c] 20 rats	Group IV LF[c] 20 rats
		Average body weight of rats/week		
21	442.4 ± 23.9	408.2 ± 19.9	440.5 ± 20.9	409.8 ± 18.1
41	536.8 ± 30.7	489.7 ± 32.7	535.5 ± 37.7	489.3 ± 24.9
63	575.8 ± 57.3	522.1 ± 34.1	574.2 ± 42.9	517.9 ± 40.3
81	561.9 ± 39.7	517.2 ± 45.3	517.2 ± 18.9	517.1 ± 37.8
98	501.0 ± 28.6[a]	457.4 ± 33.8		446.7 ± 68.0
Water consumption (liters)	9.65 ± 2.38	9.58 ± 2.04	8.75 ± 2.02	9.72 ± 2.14
Dose NNK (mg/rat)	18.1 ± 4.8	18.2 ± 4.1		
Dose NNK (mmol/kg)	0.17	0.19		
Average survival (wk)[d]	78.5 ± 15.5	86.3 ± 13.4	75.3 ± 13.0	90.0 ± 12.6
Termination (wk)	97	102	95	105

[a] Weight was taken at week 96 of the bioassay.
[b] NNK was administered as a 2 ppm solution in drinking water.
[c] The modulating effects of a high-fat *versus* low-fat diet were studied with corn oil (Table 1).
[d] The average survival of the rats in group I compared to group II was significantly shorter ($P = 0.004$) as was the average survival of rats in group III compared to those in group IV ($P = 0.0008$).

Table 3 *Tumor yields in rats treated with NNK and high-corn oil versus low-corn oil diet*

Site and type of tumor	I NNK-HF 60 rats	II NNK-LF 60 rats	III HF 20 rats	IV LF 20 rats
Total tumor-bearing rats[a]	46	40	8	7
Rats with lung tumors				
After 18 months	16[b]	3	0	0
Average size of tumors	6.8 mm²	2.5mm²		
After 24 months	30	27	1	1
Average size of tumors	19.6 mm²	17.8 mm²	3.0 mm²	4.3 mm²
Adenoma	9	7	1	0
Adenocarcinoma	20	19	0	1
Adenosquamous carcinoma	1	1	0	0
Nasal cavity				
Papilloma	5[c]	3[c]	1[c]	1[c]
Carcinoma (squamous)	0	1[c]	3[c]	1[c]
Liver				
Adenoma	3	2	0	0
Adenocarinoma	2	0	0	0

[a] Animals with tumors of the lung, pancreas, nasal cavity, and/or liver.
[b] Group I compared with Group II: $P < 0.005$. Other tumors: Leydig tumors of the testis: I, 33; II, 41; III, 14; IV, 13. Mammary tumors: I, 16; II, 7; III, 1; IV, 5. In addition, Group I had one thyroid tumor, one ear duct carcinoma, and one nephroblastoma; Group II had one intestinal adenocarcinoma; Group III had one ileum adenocarcinoma; Group IV had one thyroid carcinoma.
[c] These are tumors of the nasal mucosa related to the presence of *Aspergillus flavors* and not ethesioneuroepitheliomas like those induced by higher doses of tobacco-specific *N*-nitrosamines (21).

Thus, these bioassays demonstrate that NNK and a high-fat diet enhance and accelerate pancreas tumorigenesis. Although the final numbers of lung tumor-bearing rats were almost similar, the much earlier occurrence of lung tumors and the larger sizes of the tumors in the NNK-HF group support the human observation that cigarette smokers on a high-fat diet face an increased risk for lung cancer compared to cigarette smokers on a low-fat diet. Clearly, this study supports the epidemiological findings that high-fat diets contribute to the development of pancreas cancer in cigarette smokers.

This study raises another important question. Is the enhancement of NNK-related tumor development by the high-fat diet a consequence of the fat content of the diet *per se* and/or is it specifically due to linoleic acid, which amounts to approximately 12% of the corn oil diet? Studies at our institute had already shown that *N*-nitroso-*N*-methylurea-treated female F344 rats, maintained on safflower or corn oil (containing 82% and 56% linoleic acid, respectively), had enhanced mammary tumor yields when compared to rats of the same strain that were *N*-nitroso-*N*-methylurea-treated but maintained on olive oil or coconut oil diets which are rich in oleic and myristic acid, respectively (24). Tumors from animals that had been maintained on diets with a high linoleic acid content had high levels of prostaglandin E_2 (25). Linoleic acid is also known to stimulate the growth of human breast cells in culture (26). In nude mice, a 23% corn oil diet enhances the growth of human breast cancer cells injected into a mammary fat pad as well as their capacity to metastasize in the lungs (27). At present, we do not know whether the observed enhancing effects of dietary linoleic acid on breast tumor development in F344 rats and on lung and pancreas tumor development in the same strain of rats have common ground. One may speculate that it is so.

We are now studying the metabolism of NNK in rats on diets with various fat contents, and various fatty acids, with regard to DNA binding in the cells of the peripheral lung and of the exocrine pancreas. We also plan to determine whether a lower dose of NNK will show an even greater effect of the influence of dietary fat on the development of pancreatic and lung tumors in rats. These studies will

Table 4 *Tumors of the pancreas observed upon treatment of F-344 rats with NNK and high-corn oil versus low-corn oil diet*

	I NNK-HF (54/60)[a]	II NNK-LF (56/60)	III HF (17/20)	IV LF (19/20)
Rats with pancreas tumors				
After 18 months	11[b]	1	2	1
After 24 months	28[c]	19	6	6
Exocrine pancreas tumors				
Acinar adenoma	11	11	3	2
Acinar adenocarcinoma	6	3	3	2
Ductal adenocarcinoma	1	0	0	0
Rats with multiple tumors				
With 1 tumor	11	8	2	3
With 2 tumors	4	6	4	1
With 3+ tumors	3	0	0	0
Total no. of exocrine pancreas tumors	18	14	6	4
Average size of exocrine pancreas tumors (mm²)	17.5 ± 13.5[b, d]	9.6 ± 11.1	8.2 ± 4.2[e]	5.7 ± 3.2
Total no. of islet cell tumors	10	5	1	3

[a] Parentheses, no. of rats at risk.
[b] NNK-HF group compared with NNK-LF group: $P < 0.02$.
[c] $P < 0.05$.
[d] One rat had an uncommonly large size adenocarcinoma of the exocrine pancreas (3000 mm² cut surface at the largest diameter). To calculate the average tumor size of the exocrine pancreas tumor bearing rats in group I, its size was reduced to the next largest tumor of the group (45.5 mm²).
[e] HF group compared with LF group: $P < 0.05$.

lead to a better understanding of the risks for lung and pancreas cancer and the related mechanisms of carcinogenesis.

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