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Induction of Oral Cavity Tumors in F344 Rats by Tobacco-specific Nitrosamines and Snuff[1]

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ABSTRACT

The tumorigenic activities toward the oral cavity of snuff, its extracts, and two of its major nitrosamines, *N'*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were evaluated in male F344 rats. In one protocol, groups of 21–30 rats were treated beginning at age 10 weeks by chronic application to the oral cavity for 131 weeks of either H_2O, an H_2O extract of snuff, an H_2O extract of snuff enriched with ten times its indigenous concentration of NNN and NNK, or with NNN and NNK in H_2O. The incidence of oral cavity tumors in the rats treated with NNN and NNK was 8 of 30, compared to 0 of 30 in controls ($P < 0.05$). These results demonstrate that NNN and NNK can induce tumors locally in the oral cavity of F344 rats. Oral cavity tumors were also observed in 3 of 30 rats treated with snuff extract enriched with NNN and NNK, but not in the rats treated with snuff extract alone. In a second protocol, a test canal was surgically created in the lower lip of groups of 21–32 rats, and either snuff, H_2O-extracted snuff, or snuff enriched with its own H_2O extract was inserted in the test canal 5 times weekly for 116 weeks. A group of 10 control rats had surgery only. Among the 32 rats treated with snuff, 3 had oral cavity tumors; one was a squamous cell carcinoma originating in the test canal and invading the gingiva, one was a papilloma of the test canal, and one was a papilloma of the hard palate. Oral cavity tumors were also observed in 2 of 21 rats treated with H_2O-extracted snuff and 1 of 32 rats treated with snuff enriched with its H_2O extract. Oral tumors were not observed in control rats. The results of this study indicate that snuff and individual nitrosamines present in snuff can induce oral cavity tumors in F344 rats and support the epidemiological observations which indicate that snuff dipping causes oral cancer in man.

INTRODUCTION

Snuff dipping, the practice of placing moist snuff between the cheek and gum, has increased remarkably in the United States in recent years, particularly among adolescent males. Sales of moist snuff have increased 8 to 9% each year since 1981, reaching 39.2 million pounds in 1984. It is estimated that there are at least 7 million snuff dippers in the United States (1). According to the International Agency for Research on Cancer, "there is sufficient evidence that the oral use of snuffs of the types commonly used in North America and Western Europe is carcinogenic to humans" (2).

Commercial United States moist snuff products contain ppm quantities of carcinogenic tobacco-specific nitrosamines, ppb quantities of benzo(*a*)pyrene, and 0.2–1.2 pCi of ^{210}Po/g (3, 4). At present, the tobacco-specific nitrosamines are quantitatively the most prevalent carcinogens known in snuff. Extensive bioassays have demonstrated that two of these nitrosamines, NNN[3] and NNK, are strong carcinogens in mice, rats, and hamsters, inducing tumors of the nasal cavity, lung, esophagus, trachea, and liver (4). However, NNN and NNK have not been previously tested by application to the lips and oral cavities of rats.

The purpose of this study was to determine the ability of snuff and its components to induce tumors in the oral cavity of rats. In one protocol, groups of rats were treated by p.o. application to the oral cavity of either aqueous extracts of snuff, aqueous extracts of snuff to which 10 times the innate concentrations of NNN and NNK had been added, or aqueous solutions of NNN and NNK. In a second protocol, a test canal was surgically created in the lower lip of the rats. This allows daily insertion and retention of snuff, as described by Hirsch and Thilander (5). Groups of rats were treated by daily placement in the test canal of either snuff, the residue after H_2O extraction of snuff, or snuff enriched with its own H_2O extract.

MATERIALS AND METHODS

Preparation of Materials for Bioassays. Snuff extracts for the oral swabbing protocol were prepared from a leading commercial United States moist snuff product, purchased on the open market in 1983. The snuff was weighed and mixed with H_2O (3.3 times its weight). The mixture was stirred for 12 h with a mechanical overhead stirring unit. It was filtered and the filtrate was lyophilized. The resulting material was stored at 0–2°C until used. Solutions for group 2 of Table 1, "snuff extract," were prepared by dissolving 100 g of this residue in 200 ml of H_2O. Solutions for group 3, "snuff extract enriched with NNN and NNK," were prepared by dissolving 100 g of the residue in 200 ml of H_2O containing 27 mg of NNN and 5.5 mg of NNK. Solutions for group 4, "NNN and NNK," contained 27 mg of NNN and 5.5 mg of NNK/200 ml of H_2O. Solutions were prepared on a weekly basis and were refrigerated until used. The snuff extract was analyzed for NNN and NNK by gas chromatography with detection by a thermal energy analyzer, as previously described (6). The approximate doses (μg) given with each 0.5-ml application to the oral cavity of each rat were group 2: NNN, 6.6; NNK, 1.4; group 3: NNN, 74; NNK, 15; group 4: NNN, 68, NNK, 14.

Snuff placed in the test canal was the same brand as that used for the oral swabbing protocol. The animals in group 2 of Table 3 were treated with this snuff, which had been removed from its containers and stored in brown bottles at 0–2°C and 60% humidity. For the animals in group 3, designated "extracted snuff," 30 g snuff was weighed, mixed with 3.3 times its weight of H_2O, and the mixture was stirred for 24 h. It was filtered and the solid was air dried. The filtrate was reserved for group 4. Enough H_2O was added to the solid to bring the weight back to 30 g. This suspension was stored in brown bottles at 0–2°C until used. For group 4, "enriched snuff," 30 g of snuff was air dried. The filtrate obtained from the material prepared for group 3 was lyophilized and the residue was redissolved in enough H_2O to compensate for the amount lost during the air drying of the snuff. This material was added to the air-dried snuff and the resulting enriched snuff was stored in brown bottles at 0–2°C. The materials for groups 3 and 4 were prepared every 2 weeks. The ppm of tobacco-specific nitrosamines in these preparations were determined to be (6): group 2: NNN, 17; NNK 1.2; NAT and NAB, 19.4; group 3: NNN, 1.4; NNK, 0.9; NAT and NAB, 3.7; group 4: NNN, 28.7; NNK, 1.9; NAT and NAB, 31.8.

Bioassays. Male F344 rats, 6 weeks old, were obtained from Charles River Breeding Laboratories, Kingston, NY. They were housed in solid-bottom polycarbonate cages with hardwood bedding in groups of 3

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[3] The abbreviations used are: NNN, *N'*-nitrosonornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAT, *N'*-nitrosoanatabine; NAB, *N'*-nitrosoanabasine; HSV-1, Herpes simplex virus type 1.

under standard conditions (20 ± 2°C (SD); 50 ± 10% relative humidity; 12 h light, 12 h dark cycle). The rats were given NIH-07 diet and tap water *ad libitum*. Animals were observed until moribund, or until the experiments were terminated. The moribund animals as well as the surviving rats at the end of the experiment were sacrificed by CO_2 inhalation. Complete autopsies were performed and histology slides were prepared for all gross lesions and lungs, liver, spleen, kidney, adrenals, pancreas, esophagus, larynx, and trachea. Frontal step sections were made through the lip canal and the head (including mouth and nasal cavity). All sections were made from standard paraffin blocks with hematoxylin and eosin staining.

Statistical evaluations were carried out with the 2-sample t test and χ^2 test.

Swabbing Protocol. At 10 weeks old, the animals were divided into groups as summarized in Table 1, and each animal was treated with either 0.5 ml of H_2O (group 1), 0.5 ml of snuff extract in H_2O (group 2), 0.5 ml of NNN- and NNK-enriched snuff extract in H_2O (group 3), or 0.5 ml of NNN and NNK in H_2O (group 4). Each treatment was carried out as follows: 0.5 ml of the appropriate solution was placed in small test tubes and a sterile cotton swab was dipped into the solutions and used to swab the oral cavity and lips of each rat until the entire 0.5 ml had been used. This procedure was carried out once a day for the first 7 days, twice a day on Mondays, Wednesdays, Fridays, Saturdays, and Sundays, and once a day on Tuesdays and Thursdays of weeks 2–23, and twice each day from week 24 to week 131. The approximate total doses per rat of NNN and NNK in groups 2–4 (in μg) can be calculated as follows:

$$\text{group 2 (NNN)} = (6.6 \times 7) + (6.6 \times 264) + (6.6 \times 14\,[y - 23]) = 1{,}800 + (92\,[y - 23])$$
$$\text{group 2 (NNK)} = 380 + (20\,[y - 23])$$
$$\text{group 3 (NNN)} = 20{,}000 + (1{,}000\,[y - 23])$$
$$\text{group 3 (NNK)} = 4{,}100 + (210\,[y - 23])$$
$$\text{group 4 (NNN)} = 18{,}000 + (950\,[y - 23])$$
$$\text{group 4 (NNK)} = 3{,}700 + (190\,[y - 23])$$

where y = weeks of survival. The mean approximate total doses were: group 2 (NNN), 9,600 μg/rat (150 μmol/kg); group 2 (NNK), 2,000 μg/rat (27 μmol/kg); group 3 (NNN), 100,000 μg/rat (1,600 μmol/kg); group 3 (NNK), 21,000 μg/rat (280 μmol/kg); group 4 (NNN), 97,000 μg/rat (1,400 μmol/kg); group 4 (NNK), 19,000 μg/rat (240 μmol/kg). The assay was terminated after 131 weeks of treatment, at which time overall survival was 14%.

Test Canal Protocol. At 10 weeks old, 95 rats had surgery to create a test canal in the lower lip, as described by Hirsch and Thilander (5), with modifications as follows: a 0.2- to 0.5-cm incision was made to excise lip mucosa up to the lower incisor. After the oral mucosa was removed, both sides were sutured with stainless steel wire, 000, and buttons. The sutures were removed 7–10 days later, depending on healing. At 13 weeks old, the rats were divided into groups as summarized in Table 3. Snuff, extracted snuff, or enriched snuff was inserted in the test canal each day. Animals were restrained with a DecapiCone (Braintree Scientific, Inc., Braintree, MA) and the snuff was inserted from the oral cavity side using the cap of a Jelco catheter placement unit (Jelco Laboratories, Raritan, NJ) and a stainless steel plunger. Applications were carried out once a day except on Saturday and Sunday. Approximately 50 mg of snuff, extracted snuff, or enriched snuff was applied each time. In most animals, snuff was retained in the test canal for 24 h. Control animals underwent surgery but were given no further treatment. The assay was terminated after 116 weeks, at which time overall survival was 16%.

RESULTS

Table 1 summarizes survival and weight data for the rats treated by swabbing of the oral cavity. No significant differences in survival were observed. The weights of the rats in groups 2 and 3 were significantly lower ($P < 0.01$) than the weights of the corresponding groups not treated with snuff extract, groups 1 and 4, respectively.

Table 2 shows the incidence of tumors in the rats treated by swabbing. The incidence of oral tumors in group 4 was significantly greater than in group 1 ($P < 0.05$). Oral tumors were also observed in group 3 but their incidence was not significantly greater than that in group 1. No other significant differences ($P < 0.05$) were detected among the groups. However, the occurrence of 4 lung adenocarcinomas in group 4 should be noted.

Table 3 summarizes survival and weight data for the rats treated by insertion of snuff into a test canal in the lower lip. Mean survival was not significantly different among the 4 groups. The weights of the animals in groups 2–4 were significantly ($P < 0.05$) lower than those in group 1 through treatment week 84. After week 84, significant weight differences were not observed.

Table 4 shows tumor incidence in the rats treated according to the test canal protocol. Tumors of the oral cavity and nasal cavity were observed only in the animals treated with snuff. Significant differences in tumor incidence among the groups were not observed.

The oral tumors which developed in both protocols were

Table 1 *Survival and weights of male F344 rats treated with snuff extracts or NNN and NNK*

The oral cavities of rats were swabbed with 0.5 ml of either H_2O, an H_2O extract of snuff, an H_2O extract of snuff enriched with NNN and NNK, or with NNN and NNK in H_2O. For details, see "Materials and Methods."

Group	No. of rats	Survival (wk)	Wt (g) at wk 26	53	78	104
1. H_2O control	21	103 ± 34[a]	350 ± 18	404 ± 25	420 ± 27	404 ± 33
2. Snuff extract	30	108 ± 24	330 ± 15	363 ± 17	371 ± 18	354 ± 22
3. Snuff extract enriched with NNN and NNK	30	106 ± 32	317 ± 18	358 ± 18	366 ± 19	362 ± 25
4. NNN and NNK	30	106 ± 39	350 ± 17	405 ± 18	427 ± 28	385 ± 35

[a] Mean ± SD.

Table 2 *Tumor incidence in male F344 rats treated with snuff extracts or NNN and NNK*

The oral cavities of rats were swabbed with 0.5 ml of either H_2O, an H_2O extract of snuff, an H_2O extract of snuff enriched with NNN and NNK, or with NNN and NNK in H_2O. For details, see "Materials and Methods."

Group	No. of rats[b]	No. of rats with tumors[a]: Oral cavity	Lungs	Prostate[c]	Mammary	Leukemia or lymphoma
1. H_2O control[d]	21	0	1[e]	5	7[f]	11
2. Snuff extract[g]	30	0	0	9	8[h]	4
3. Snuff extract enriched with NNN and NNK[i]	30	3[j]	2[e]	6	3[k]	6
4. NNN and NNK[l]	30	8[m]	5[n]	11	7[o]	9

[a] 80–90% of rats in all groups had interstitial testicular tumors.
[b] Complete necropsy was performed on all rats.
[c] Carcinoma *in situ*.
[d] Other tumors; 1 liver adenoma, 1 heart fibrosarcoma, 1 thyroid adenoma, 1 thyroid adenocarcinoma, 1 pancreas islet tumor.
[e] Adenoma.
[f] Three fibroadenomas, 4 adenocarcinomas.
[g] Other tumors: 1 skin papilloma, 1 spleen fibroma, 1 larynx adenoma, 3 peritoneal mesotheliomas, 1 thyroid adenoma, 2 thyroid adenocarcinomas, 2 pancreas islet tumors, 3 preputial gland adenomas, 2 kidney pelvis papillomas.
[h] Two adenomas, 1 fibroma, 5 fibroadenomas.
[i] Other tumors: 1 peritoneal mesothelioma, 1 pancreas islet tumor, 1 preputial gland adenoma, 1 preputial gland adenocarcinoma, 1 skin adenoacanthoma, 1 ear duct tumor, 1 kidney pelvis papilloma.
[j] One cheek papilloma, 1 hard palate papilloma, 1 tongue papilloma.
[k] One adenoma, 1 fibroadenoma, 1 fibroma.
[l] Other tumors: 1 colon carcinoma, 1 cecum carcinoma, 1 peritoneal mesothelioma, 2 thyroid adenomas, 3 thyroid adenocarcinomas, 3 pancreas islet cell tumors, 2 ear duct tumors.
[m] Six cheek papillomas, 1 hard palate papilloma, 2 tongue papillomas.
[n] One adenoma, 4 adenocarcinomas.
[o] One adenoma, 3 fibroadenomas, 3 adenocarcinomas.

Table 3 *Survival and weights of male F344 rats treated by application of snuff in a test canal*

Snuff, H_2O-extracted snuff, or snuff enriched with its own H_2O extract was inserted 5 times weekly in a surgically created test canal in the lower lip of each rat. Control rats were treated with surgery only. For details, see "Materials and Methods."

Group	No. of rats	Survival (wk)	Wt (g) at wk 28	52	76	104
1. Control	10	104 ± 10[a]	414 ± 15	482 ± 23	479 ± 35	408 ± 66
2. Snuff	32	108 ± 10	386 ± 16	440 ± 23	445 ± 27	418 ± 28
3. Extracted snuff	21	104 ± 13	379 ± 16	436 ± 21	452 ± 29	419 ± 22
4. Enriched snuff	32	101 ± 12	384 ± 16	440 ± 22	453 ± 29	425 ± 83

[a] Mean ± S.D.

mostly squamous cell papillomas arising from the squamous stratified epithelium lining of the lips, cheeks, roof and floor of the mouth, as well as the tongue. All tumors were large and exophytic occupying a portion of the oral cavity. The squamous cell carcinoma reported in group 2 of Table 4 originated in the tissues of the test canal and infiltrated deeply into the lip tissue and the mandibular gingiva (see Figs. 1–4).

Nonspecific lesions of the oral cavity including hyperkeratosis, acanthosis, chronic or acute inflammation, and granulomas were also observed in both protocols, independent of the tumors, but these were not quantified in the present study.

Table 4 *Tumor incidence in male F344 rats treated by application of snuff in a test canal*

Snuff, H_2O-extracted snuff, or snuff enriched with its own H_2O extract was inserted 5 times weekly in a surgically created test canal in the lower lip of each rat. Control rats were treated with surgery only. For details, see "Materials and Methods."

Group	No. of rats[b]	No. of rats with tumors[a] Oral cavity	Nasal cavity	Forestomach[c]	Lungs[d]	Liver[e]	Pancreas	Prostate	Mammary	Leukemia or lymphoma
1. Control	10	0	0	1	1	1	0	3	1	3
2. Snuff	32	3[f]	0	1	2	2	2	2	6	7
3. Extracted snuff	21	2[g]	0	1	2	3	2	4	4	3
4. Enriched snuff	32	1[h]	1[i]	2	2	6	2	4	1	7

[a] 80–90% of rats in all groups had interstitial testicular tumors.
[b] Complete necropsy was performed on all rats.
[c] Small papillomas.
[d] Adenomas, except in group 1 which was a metastasis of an osteosarcoma.
[e] Hyperplastic nodules.
[f] Two rats had tumors in the test-canal; 1 was an early papilloma and 1 was a squamous cell carcinoma invading the gingiva. One rat had a hard palate papilloma.
[g] One rat had a tongue papilloma and 1 had a hard palate papilloma.
[h] Papilloma of the floor of the mouth.
[i] Olfactory tumor (esthesioepithelioma).

Fig. 1. Squamous papilloma of the tongue in a rat treated with NNN and NNK. H & E.

Fig. 2. Squamous cell carcinoma in the test canal of the lip in a rat treated with snuff. H & E.

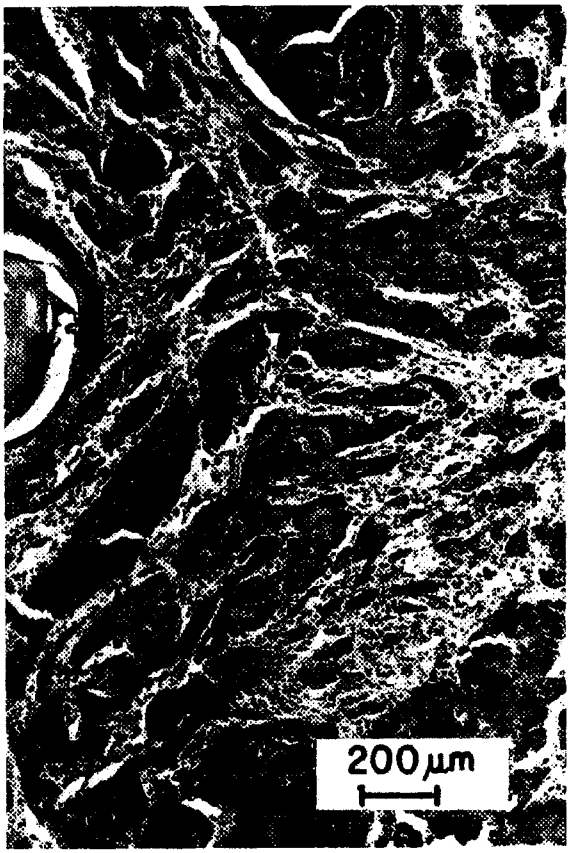

Fig. 3. Enlarged area of Fig. 2. Squamous cell carcinoma deeply infiltrating the stroma. H & E.

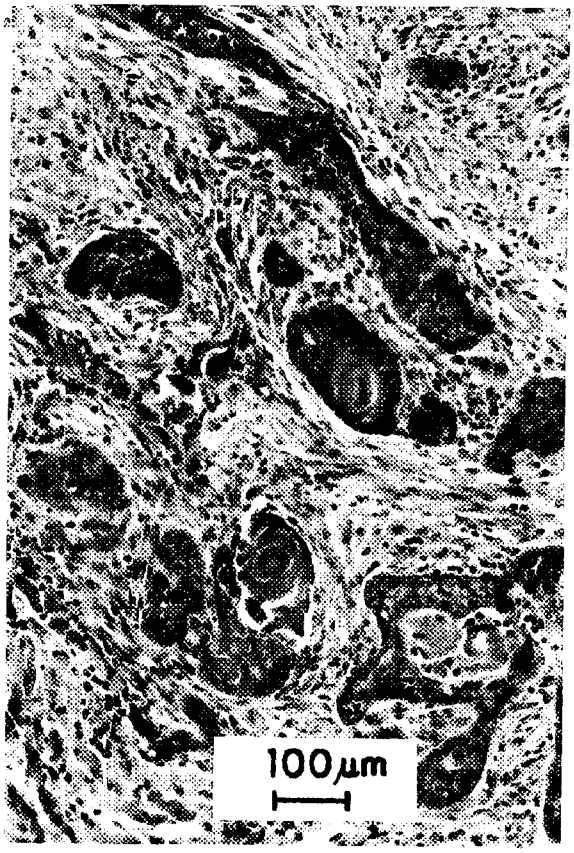

Fig. 4. Gingiva of lower maxillary, infiltrated by the squamous cell carcinoma illustrated in Fig. 2.

DISCUSSION

The results of the bioassay carried out with the swabbing protocol clearly show that a mixture of NNN and NNK can induce tumors in the oral cavity of F344 rats. These results are significant because NNN and NNK are the major known carcinogens in snuff. Epidemiological studies have shown that snuff induces oral tumors in man (1, 2, 4).

Previous bioassays of NNN have shown that, in contrast to many nitrosamines, its carcinogenicity depends on the route of administration. NNN given to F344 rats in the drinking water or in a liquid diet induces a high incidence of esophageal and nasal cavity tumors, but when given by s.c. injection it induces almost exclusively nasal cavity tumors (7–12). These findings suggested that NNN could act locally as well as systemically and the present data appear to support that suggestion. Bioassays of NNK given in the drinking water have not been reported.

The induction of 4 lung adenocarcinomas in the group treated with NNN and NNK is likely to be related to treatment despite its lack of statistical significance. Previous studies have shown that NNK, administered s.c. to F344 rats (total dose, 0.3 mmol/kg) induced adenocarcinomas in 4 of 27 animals (13). In contrast, the absence of nasal cavity tumors in the group treated with NNN and NNK was surprising because it was a major target tissue when comparable doses of these compounds were administered s.c. to F344 rats (12, 13).

Whereas the incidence of oral cavity tumors was 8 of 30 in the rats treated with NNN and NNK, it was only 3 of 30 in the rats treated with snuff extract enriched with NNN and NNK. This difference was not statistically significant, but taken together with the negative results obtained in the animals swabbed with snuff extract only, it suggests that NNN and NNK were less tumorigenic when administered together with snuff extract than when administered alone. This might in part be associated with the significantly lower weights of the animals in groups 2 and 3 compared to those in group 4, suggesting a general toxic effect of snuff extract, perhaps due to nicotine. It is also possible that nicotine, which is present in great excess over NNN and NNK even in the enriched extract, might act as a competitive inhibitor of their metabolic activation in the oral mucosa. Furthermore, snuff extract may contain other inhibitors of NNN and NNK activation, as shown for a number of other plant-derived compounds (14, 15). Studies are required to elucidate the mechanism of the apparent inhibitory effect of snuff extract on NNN and NNK tumorigenesis in the rat oral cavity.

The lack of oral tumors in the animals treated with snuff extract indicates that this mixture is not tumorigenic in the rat oral cavity, when the swabbing protocol is used. Previous bioassays of snuff extracts, applied to the oral cavities of rats, mice, and hamsters have also generally yielded negative results (1). However, application to the lips of mice of snuff extract together with HSV-1 did result in epithelial dysplasia and other histomorphological changes (16). Considering the fact that the concentrations of NNN and NNK in the snuff extract used to treat group 2 were 0.1 times their concentrations in the extract used for group 3, the lack of tumorigenicity of the snuff extract in this model is not surprising.

The results of the bioassay of snuff with the test canal protocol were similar to those reported by Hirsch and Johansson (17), who placed approximately 0.2 g of a Swedish snuff product into the test canals of 42 Sprague-Dawley rats twice a

day, 5 days a week for 9–22 months. One macroscopic tumor of the oral cavity was detected. It was ulcerated and situated on the left side of the oral cavity, extending from the incisors and involving both the upper and lower maxillae. It was a moderately well-differentiated squamous cell carcinoma invading the bone. In the present study, one rat had a squamous cell carcinoma of the test canal invading the gingiva, one rat had a papilloma of the test canal, and one rat had a squamous cell papilloma of the hard palate, among the 32 rats treated with snuff. While this incidence rate was not statistically significant compared to controls, the occurrence of spontaneous tumors of the oral cavity in F344 rats does not exceed 0.05% (7–13, 18, 19). Taken together with the results from groups 3 and 4, in which oral tumors were observed in 3 rats, these findings strongly indicate that these tumors were induced by snuff and were not fortuitous occurrences. In addition, the oral tumors originated in the squamous epithelium, as observed in humans.

The occurrence of one nasal olfactory tumor in the group treated with enriched snuff is also of interest since this tumor type is rare or nonexistent in control F344 rats (7–14, 18, 19). The induction of nasal tumors would be expected in animals treated with mixtures containing NNN and NNK, although as discussed above this was not observed in the swabbing protocol.

In designing the protocol for the test canal experiment, we wished to test the hypothesis that NNN and NNK, as present predominantly in the H_2O extract of snuff, are responsible for its tumorigenic effects. Therefore, we included groups 3 and 4 in which the levels of these nitrosamines were lower or higher than in group 2. Unfortunately, the relatively low tumor incidence in groups 2–4 prevent an evaluation of this hypothesis. The low tumor incidence in group 4 could result from the presence in the extract of inhibitors of NNN and NNK tumorigenesis, as was apparent from the swabbing protocol. In retrospect, a better design might have included a group in which only NNN and NNK were added to snuff or to extracted snuff.

In contrast to the relatively low tumor incidences observed in the rats treated with snuff or its extracts in the present study, a recent report has demonstrated the production of epithelial dysplasia and squamous carcinoma in the buccal pouches of 11 of 25 Syrian golden hamsters treated with snuff and HSV-1 (20). Treatment of HSV-1-infected rats with snuff by the test canal protocol resulted in oral tumors in 2 of 7 rats compared to incidences of 0 of 7 or 0 of 10 in animals treated only with snuff or HSV-1 (21). Replication of HSV-1 is inhibited by snuff extracts and in particular those with high levels of tobacco-specific nitrosamines (22). It would be important to determine whether HSV-1 infection could enhance the observed tumorigenicity of NNN and NNK in the oral cavity. The tumorigenicity of NNN and NNK in the oral mucosa might also be enhanced by chronic irritation as has been observed for *N*-nitroso-*N*-methylurea (23).

In conclusion, this study has demonstrated that snuff and tobacco-specific nitrosamines can induce tumors in the rat oral cavity. These findings are significant because they support the epidemiological observations which indicate that snuff dipping causes oral cancer in man (1, 2). The data indicate, however, that components of snuff extract may inhibit tumor induction by tobacco-specific nitrosamines.

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