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EXPERIMENTAL STUDIES INVOLVING EXPOSING LABORATORY ANIMALS TO SMOKELESS TOBACCO OR ITS CONSTITUENTS

This section reviews bioassays evaluating the carcinogenicity in animals of smokeless tobacco and its constituents, particularly the tobacco-specific nitrosamines (TSNA) described in the section on the chemical constituents of smokeless tobacco. The bioassays involved multiple routes of administration of chewing tobacco, snuff, or extracts of these products and of several TSNA.

Studies of chewing tobacco, snuff, and TSNA are summarized in tables 1 to 3 respectively, with comments on the individual investigations provided below.

Bioassays With Chewing Tobacco

Oral Administration

An alcohol extract of Indian chewing tobacco diluted 1:50 (group 1) or 1:25 (group 2) was gavage-fed to male Swiss mice over 15 to 20 months. In another group of mice, a mixture of the tobacco extract with standard laboratory diet was administered over 21 to 25 months (group 3). This treatment produced tumors in 8 of 15 mice at risk in group 1, including 5 mice with lung tumors and 2 with liver tumors; 4 of 10 mice at risk in group 2 developed lung and liver tumors. The feeding experiment (group 3) resulted in 8 of 10 mice with tumors, specifically 4 with tumors of the lung and 4 with liver tumors. Despite the high toxicity of the tobacco extracts and certain short-comings of the methodology, these assays indicate that the extract of chewing tobacco is carcinogenic in mice *(1).*

Application to the Oral Mucosa and Cheek Pouch

Three different extracts of an Indian chewing tobacco were applied daily for up to 18 months to the buccal mucosa of strain A and Swiss mice. No excess of tumors was observed *(2).* The oral mucosa of a group of weanling Wistar rats was painted twice weekly with a 2-percent alkaloid-free extract of an Indian chewing tobacco. No tumors were observed at the application site even though applications were continued throughout the lifespan of the rats *(3).*

TABLE 1.—Bioassays for Carcinogenic Activity of Chewing Tobacco or Chewing Tobacco Extracts*

Route of Application	Species, Sex	Test Material and Dose	Duration of Exposure (Months)	Fraction of Animals With Tumors		Reference
				Exposed	Controls	
Oral-intubation	mice, M	extract diluted 1:25 diluted 1:50	4/1/2 15-20	4/10† lung adenocarcinoma 8/15† and liver carcinoma	0/20	*1*
Oral-feeding	mice, M	0.2% extract in diet	21-25	8/10† lung adenocarcinoma	1/20	*1*
Skin-topical	mice, M+F	DMSO extract (dose ?)	21-22	0/10 0/7		
Oral-swabbing	mice, M+F	extracts applied daily, dose not given	up to 18	no excess tumors compared to controls		*2*
Oral-swabbing	rats (NS)‡	2% alkaloid-free extract, dose not given- + lime	Lifespan	0/10 0/12	0/10 0/14	*3*
Oral-pouch implantation	hamsters (NS)	2-cm^3 plug	up to 30	0/50		*7*
Oral-pouch	hamsters (NS)	DMSO-extract three times weekly	18-24	0/12	0/7	*5*
Oral-pouch swabbing	hamsters, M	DMSO-extract three times weekly, dose not given	5	0/12	0/11	*4*
Oral-pouch swabbing	hamsters, F	2% tobacco extract in water, twice daily application	6	3/17	0/10	*6*
Subcutaneous injection	mice (NS)	2% tobacco extract partially or completely free of alkaloids, 25 solution once a month	10-23	1/17 squamous-cell carcinoma (site not specified)		*8*

* Abbreviation: DMSO, dimethyl sulfoxide.

† Animals at risk.

‡ (NS) = not stated.

TABLE 2.—Bioassays for Carcinogenic Activity of Snuff or Snuff Extracts*

Route of Application	Species, Sex	Test Material and Dose	Applications	Duration of Exposure (Months)	Fraction of Animals With Tumors		Reference
					Exposed	Controls	
Oral-feeding	Hamsters, M	S, 20% of diet	Once daily	24	0/100†	0/100	*17*
Lips-painting	Mice, M	SE, dose not given	3 times daily	2	0/20	0/20	*18*
Oral-swabbing	Rats, M	SE+H	0.5 ml daily	2	0/20	0/20	*18*
		SE (approx. 30%)	0.5 ml daily	up to 30	0/30	1/21 (lung adenoma)	*20*
		SE (approx. 30%) + (NNN+NNK)	0.5 ml daily	up to 30	5/30 (3 papilloma in oral cavity, 2 lung adenoma)	1/21 (lung adenoma)	*20*
		NNN+NNK	0.5 ml daily	up to 30	13/30 (8 papilloma in oral cavity, 5 lung adenoma)	1/21 (lung adenoma)	*20*
Lip canal-instillation	Rats, F	S	200 mg twice daily	9-22	1/42† (oral carcinoma)	0/20	*21*
		S	200 mg twice daily	18	1/10 (oral carcinoma)	0/10	22
		H	200 mg	18	0/7	0/10	*22*
		S+H	twice daily	18	2/7 (2 oral carcinoma)	0/10	*22*
Lip canal-instillation	Rats, M	S	50 mg daily	up to 30	3/32 (papilloma and 1 carcinoma in test canal, 1 oral papilloma)	0/10	*20*
		S+Se	50 mg daily	up to 30	1/32 (oral papilloma)	0/10	*20*
		ES	50 mg daily	up to 30	2/21 (oral papilloma)	0/10	*20*
Cheek pouch-instillation	Hamsters (NS)‡	S	10 ml paste once	up to 30	0/50	0/50	*7*
		S	?	6	0/25		*27*
		H	?	6	0/25		*27*
		S+H	?	6	11/25 (papilloma and carcinoma of the oral cavity)		*27*
Subcutaneous injection	Rats, M+F	SE	50 mg, 84 weekly applications	26	0/82	0/82	*28*
	Rats (NS)	TE	45 mg, 70 weekly applications	21 ± 4	18/75	1/75	*29*

* Abbreviations: ES, extracted snuff; H, infected with herpes simplex virus; NNK, 4-(methylnitrosamino)-1-(-3-pyridyl)-1-butanone; NNN, N'-nitrosonornicotine; S, snuff; SE, snuff extract.
† No tumors of the oral cavity, esophagus, nasopharynx and larynx; all other tumors nearly identical to those in control animals.
‡ (NS) = not stated.

TABLE 3.—Carcinogenicity of Tobacco-Specific Nitrosamines*

Nitrosamine	Species and Strains	Route of Application	Principal Target Organs	Dose
NNN	A/J mouse	i.p.	lung	0.12 mmol/mouse
	F344 rat	s.c.	nasal cavity esophagus	0.2-3.4 mmol/rat
		p.o.	esophagus nasal cavity	1.0-3.6 mmol/rat
	Sprague-Dawley rat	p.o.	nasal cavity	8.8 mmol/rat
	Syrian golden hamster	s.c.	trachea nasal cavity	0.9-2.1 mmol/hamster
NNK	A/J mouse	i.p.	lung	0.12 mmol/mouse
	F344 rat	s.c.	nasal cavity lung, liver	0.1-2.8 mmol/rat
	Syrian golden hamster	s.c.	trachea, lung, nasal cavity	0.9 mmol/hamster 0.005 mmol/hamster
NAT	F344 rat	s.c.	none	0.2-2.8 mmol/rat
NAB	F344 rat	p.o.	esophagus	3-12 mmol/rat
	Syrian golden hamster	s.c.	none	2 mmol/hamster
NNA	A/J mouse	i.p.	none	0.12 mmol/mouse

* Hoffmann and Hecht *(11)*.

A group of 12 male Syrian golden hamsters received topical applications on the buccal mucosa of a dimethyl sulfoxide (DMSO) extract of an Indian chewing tobacco three times weekly for 21 weeks. None of the treated hamsters developed tumors in the oral mucosa; however, 8 of 12 treated animals had leukoplakia. These changes were not seen in the oral mucosa of the animals treated with DMSO alone *(4)*. In another bioassay, 12 male hamsters received applications to the cheek pouch of a DMSO extract of Indian chewing tobacco three times weekly over their entire lifespan. Tumors were not observed in the treated group or the control group *(5)*. When 1 mg of a paste made of a chewing tobacco extract was applied topically to the mucosa of the cheek pouches twice daily over a 6-month period, and animals were maintained without further treatment for another 6 months, the incidence of hyperplasia in the buccal pouches was 17.6 percent, that of dysplasia was 29.4 percent, and that of squamous cell papilloma or carcinoma was 17.6 percent in 17 hamsters. There were no tumors in the 20 control animals *(6)*.

Fifty hamsters received implantations of a 2 cm^3 plug of chewing tobacco in their cheek pouches. The opening of the cheek pouch was ligated and the animals were observed for 18 months. After 13 months, 21 of 50 animals had survived. No tumors were recorded upon termination of the assays *(7)*.

Although the studies cited above had some inherent weaknesses due to short application time or low dose, it appears, nevertheless, that both the oral mucosa of rats and the cheek pouches of Syrian golden

hamsters are relatively resistant to the carcinogenic activity of the extracts of chewing tobacco.

Subcutaneous Application

Seventeen C57 black mice were subcutaneously injected with 1 ml of a 2-percent solution of either partly or completely alkaloid-free extracts of an Indian chewing tobacco once a month for 1 to 24 months. One squamous carcinoma at an unspecified site developed in one mouse receiving the partly alkaloid-free extract *(8)*.

Skin Application

A large number of studies have been published regarding the tumorigenicity on mouse skin of various extracts of chewing tobacco. Most of these bioassays failed to produce skin tumors. The negative results appear to be due primarily to the low dose applied or the short duration of the applications *(9,10)*. The negative results indicate also that the concentrations of TSNA and PAH in these extracts do not suffice to induce tumors upon topical application *(11)*. However, the application of methanol or DMSO extracts of cigarette tobacco induced a low but significant number of benign tumors in the skin of CAF1 and Swiss mice when these extracts were applied three times weekly for up to 24 months to the shaved backs of the mice *(12,13)*. A number of studies have reported tumor-promoting activity of the extracts of chewing tobacco when these were applied to mouse epidermis previously treated with a tumor initiator *(8,12,14-16)*. The bioassay data with chewing tobacco are summarized in table 1.

Bioassays With Snuff

Oral Administration

For 2 years, 50 male BIO 15.16 and 50 male BIO 87.20 hamsters were each maintained on a standard diet containing 20 percent moist, fresh snuff. Controls consisted of 50 male BIO 15.16 hamsters and 50 male BIO 87.20 hamsters on a diet containing 20 percent cellulose (of caloric value similar to the snuff-containing diet). The spectrum of tumors observed was nearly identical in both groups. Hamsters of both strains gavaged 60 times with 5 mg of the carcinogen 3-methylcholanthrene (MC) had a significantly increased incidence of both benign and malignant tumors of the forestomach and large intestine. Hamsters of the BIO 87.20 strain also had an increased incidence of stomach cancers while the BIO 15.16 strain developed tumors of the skin. To assay the cocarcinogenic activity of snuff, 50 hamsters of each strain received the diet containing 20 percent snuff plus 50 times 0.5 mg of MC. Compared to the control group (diet containing 20 percent cellulose), the tumor yield was not increased in the two experimental groups indicating a lack

of carcinogenic activity as well as of cocarcinogenic activity of the snuff in this setting *(17)*.

Application to the Lip, Oral Mucosa, or Cheek Pouch

The upper lips of 20 male BALB mice were painted 3 times a day for 5 days weekly over a 2-month period with a concentrated water extract of snuff (group 1). In another group of 20 male mice, the upper lips were inoculated with herpes simplex virus type 1 (HSV-1) and were subsequently painted with a concentrated snuff extract for 2 months (group 2). A control group of 20 male mice received inoculation of the upper lips with HSV-1 and painting with water (group 3). Two months' exposure to snuff extract (group 1) or HSV-1 inoculation (group 3) alone did not induce dysplasia in the epithelium of the labial mucosa, while HSV-1 inoculation combined with painting of snuff extract produced epithelial dysplasia and other histomorphologic changes *(18)*.

In respect to this and other studies in which animals are infected with herpes virus in addition to treatment with snuff extracts, it should be noted that 20 to 40 percent of the U.S. population have periodic occurrences of labial herpes *(19)*.

Male F344 rats were treated for up to 30 months by swabbing the oral cavity with either a concentrated water extract of snuff (group 1; 13.2 μg NNN and 2.8 μg NNK per milliliter snuff extract solution), snuff extract enriched with the tobacco-specific nitrosamines NNN and NNK (group 2; 148 μg NNN and 30 μg NNK per milliliter snuff extract solution), NNN and NNK alone in concentrations corresponding to those applied in group 2 (group 3; 135 μg NNN and 27.6 μg NNK per milliliter test solution), or with water alone (group 4). Groups 1, 2, and 3 consisted of 30 male rats each and group 4 (control) of 21 rats. The incidence of tumors in groups 1 and 2 was not significantly increased over that in the control group. In the group of 30 rats treated with NNN and NNK alone, 8 animals had oral tumors (6 papillomas in the cheek, 4 papillomas in the hard palate, and 1 papilloma of the tongue), and 4 animals had lung carcinoma. This study indicates that snuff contains carcinogenic N-nitrosamines; however, when they are being tested in an admixture with other components in the water extract of snuff, their carcinogenic activity may be suppressed *(20)*.

A group of 21 male and 21 female Sprague-Dawley rats were treated with snuff placed in a surgically created canal in the lower lip. Approximately 0.2 g of a standard Swedish snuff (pH 8.3) was given twice daily 5 days per week for 9 to 22 months. The mean retention time of the snuff in the canal was 6 hours, and the estimated daily dose was 1 g of snuff/kg b.w. Using the same methodology, another group of 5 male and 5 female rats was treated with alkaline snuff in the surgically created canal (pH 9.3). One of the 42 rats treated with regular snuff developed a squamous carcinoma in the oral cavity after 8.5 months. The exposure to the regular snuff resulted in mild to moderate hyperplasia of the epithelium,

hyperorthokeratosis, and acanthosis. Among rats exposed to snuff for 18 to 22 months, 16 of 42 showed vacuolated cells penetrating deeper into the epithelium with hyperplastic and atropic lesions. Rats exposed to alkaline snuff differed little from those in the group treated with regular snuff. Outside the area of treatment, squamous cell hyperplasia of the forestomach was found in rats exposed to snuff for 18 to 22 months *(21)*.

In another bioassay using the same methodology as described by Hirsch and Johansson *(21)*, the surgically created canal in the lower lip of F344 rats was filled five times each week over 28 months with either U.S. snuff (average 0.2 g per application; n=30), snuff enriched with its own water extract (n=30), or the extracted residue of snuff (n=21). Ten rats with the surgically created lip canal, and otherwise untreated, served as controls. The incidence of nonspontaneous tumors in each group was the following: rats treated with snuff had one squamous carcinoma of the oral cavity, one squamous cell papilloma of the hard palate, and one meningioma; treatment with enriched snuff led to one squamous cell papilloma of the floor of the mouth and one nasal olfactory tumor; treatment with extracted snuff induced one squamous cell papilloma of the hard palate. There were no tumors in the control group *(20)*.

Four groups of female Sprague-Dawley rats with surgically created canals in the lower lip, received the following treatments beginning at 3 months of age: group 1 was infected with herpes simplex virus type 1 (HSV-1) by scarification and topical application followed 10 days later by administration of snuff into the canal morning and night on 5 days per week; group 2 was infected with virus and received no other treatment; group 3 was sham-infected with sterile saline followed by snuff treatment; and group 4, not given virus or snuff, served as controls. The HSV-1 infection was repeated once after a 1-month interval, and snuff treatment was continued for 18 months after which time all animals were killed. Three animals in each of groups 1 and 2 died from encephalitis shortly after the second infection with HSV-1. Squamous-cell carcinomas of the oral cavity developed in two of seven rats, and a retroperitoneal sarcoma was seen in one of seven rats exposed to HSV-1 plus snuff. In the group exposed to snuff alone, 1 of 10 animals developed a squamous carcinoma of the anus and 1 of 10 a retroperitoneal sarcoma *(22)*.

In several studies, various forms of snuff were installed in the cheek pouches of Syrian golden hamsters for up to 20 months. The application of snuff did not lead to the induction of tumors in the cheek pouches nor at any other site of the oral cavity in any of these studies even though malignant tumors were induced in the oral cavity with high doses of 7, 12-dimethylbenz(a) anthracene and 3-methylcholanthrene *(7,23-26)*.

In an assay for the joint action of HSV and snuff, the buccal pouches of 125 Syrian hamsters were inoculated with HSV-1, HSV-2, or culture medium. The control and HSV inoculations were done once a month for 6 consecutive months. Then 25 hamsters with HSV-inoculated pouches received installations of commercial snuff twice daily into both the right

and left pouches. One month after the last HSV inoculation and 6 months after continuous snuff application, the assay was terminated. The buccal pouches were removed for histopathologic examination. Neither the application of snuff to the cheek pouches nor HSV infection alone induced neoplastic changes in hamster buccal pouches. However, HSV infection in combination with snuff resulted in epithelial dysplasia and in squamous carcinoma of the buccal pouches in 11 out of 25 hamsters *(27)*. This investigation provides the strongest evidence to date that snuff may increase cancer risk in animals; however, full evaluation is precluded since the findings have been published only in abstract form.

Subcutaneous Administration

A Swedish snuff was extracted with 60-percent alcohol and resulted in 18-percent dry extract, which was injected subcutaneously into rats with 70-percent ethanol and tri-n-caprylin (1:1) as vehicle. The rats received a total dose of 4.2 g of extract with 84 weekly doses of 50 mg of extract. No tumors were observed at the area of injection *(28)*. This result is quite different from an earlier one by the same investigators in which an alcohol extract from cigarette tobacco (20-percent yield) was injected into 75 rats with 70-percent alcohol and glycerol as solvent (1:3). Per week, 45 mg extracts were injected until the total dose amounted to 3.2 g/rat. After 25 months, 18 of 75 rats had developed malignant tumors, primarily sarcomas at the injection site *(29)*. The bioassay data with snuff are summarized in table 2.

Bioassays With Constituents of Smokeless Tobacco

At least three types of carcinogens occur in smokeless tobacco: polynuclear aromatic hydrocarbons (PAH), polonium-210 (^{210}Po), and N-nitrosamines. One of the PAH identified in smokeless tobacco, benzo(a)pyrene (up to 72 ppb), has long been recognized as an animal carcinogen *(18,24,30)*. Levels of ^{210}Po in processed tobacco amount to 0.1-1.0 pCi per gram and to 0.18-1.22 pCi/g in commercial U.S. snuff products. Ionizing radiation can cause multiple types of cancer in animals and humans raising the possibility that the alpha-radiation of ^{210}Po may contribute to the carcinogenic potential of smokeless tobacco and especially snuff *(31,31)*.

Three groups of N-nitrosamines have been identified in smokeless tobacco. All of the 4 volatile nitrosamines thus far identified are carcinogenic in animals *(33)*. These are nitrosodimethylamine (0 to 215 ppb), nitrosopyrrolidine (0 to 291 ppb), nitrosopiperidine (0 to 107 ppb), and nitrosomorpholine (0 to 690 ppb). Seven nonvolatile nitrosamines have also been identified in smokeless tobacco. Of these, only nitrosodiethanolamine (30 to 6,800 ppb) is a known carcinogen in mice, rats, and hamsters *(33)*. Swabbing of the oral cavity of 20 male and 20 female hamsters with solutions of these agents three times weekly for 45 weeks

(20 mg per application) induced tumors of the nasal cavity in 17 animals, tumors of the trachea in 6, and a tumor of the larynx in 1 of the hamsters *(34)*.

The most abundant carcinogens in smokeless tobacco yet identified are the tobacco-specific nitrosamines (TSNA). These are formed during the processing of tobacco from its alkaloids. So far, seven TSNA have been identified in smokeless tobacco. Of these, N'-nitrosonornicotine (NNN; 470-135,000 ppb) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; 30-13,600 ppb) are powerful carcinogens in mice, rats, and hamsters (table 1; 1,9). Table 3 summarizes results from bioassays administering TSNA to test animals. A variety of tumors were produced, particularly in the esophagus, nasal cavity, and lung. In a recently completed investigation, daily swabbing for up to 30 months of the oral cavity of F344 rats with a saline solution containing 135 ppm NNN and 28 ppm NNK led to the development of benign oral tumors in 8 and lung carcinoma in 4 of 30 rats. Neither oral tumors nor tumors of the lung were observed in the negative control group *(20)*. This study suggests that NNN and NNK may be tumorigenic at the site of exposure as well as systemically. Full evaluations of these results are precluded, however, since the original manuscript is now under journal review and not published.

It is noteworthy that some of the bioassays indicated that relatively low doses of the TSNA could induce tumors. In hamsters, a total dose of only 0.2 mmol/kg of NNK induced a significant incidence of tumors *(35)*, whereas in F344 rats, 60 subcutaneous injections of a total dose of 20 mg (0.33 mmol/kg) of NNK induced tumors of the liver in 10, tumors of the lung in 13, and tumors of the nasal cavity in 6 of 30 rats. Subcutaneous applications to 27 rats of the same molar dose (0.33 mmol/kg) of nitrosodimethylamine resulted in 6 animals with tumors of the liver and 1 rat with a tumor of the nasal cavity *(36)*. For NNN, high tumor incidences were produced in F344 rats by a total dose of 1.0 mmol/kg *(37)*. Based on daily use for 30 years of 10 g of snuff containing 3.1 ppm of NNK, the estimated NNK exposure of a snuff dipper would be approximately 0.02 mmol/kg. Exposure to NNN from the same brand would be 0.4 mmol/kg (figure 3, chapter 2). Hence, the bioassays indicate that exposures in the dose range actually experienced by long-term snuff dippers induce tumors in animals. This is a distinctive and potentially important finding, since for most chemical carcinogens their carcinogenicity was detected following exposure at doses much higher than usually received by humans.

Of the other five TSNA, besides NNN and NNK, N'-nitrosoanabasine (NAB; 10-6,700 ppb) and 4(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL; 140-300 ppb) were moderately active carcinogens, and N-nitrosoanatabine (NAT; 300-338,000 ppb) was inactive when tested at the low dose level of 9 mmol/kg *(9,38)*.

Recently, two additional TSNA have been identified in snuff: 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanone (1,300-1,800 ppb) and 4-(methylnitrosamino)-(3-pyridyl)butene-1 (10 ppb; 6). These two nitrosamines have not yet been tested for carcinogenicity.

Mutagenicity Assays and Other Short-Term Tests

Chewing Tobacco

Nicotiana rustica is a tobacco variety that is widely cultivated and used throughout India. Its ethanol extracts induced mutations in *Salmonella typhimurium* TA98 and in V79 cells of Chinese hamsters. The addition of S9 liver homogenate from Aroclor-pretreated rats enhanced the mutagenic effect. No mutations were induced in TA100, TA1535, or TA1538 in the presence of the S9 homogenate. This ethanol extract of tobacco also induced micronuclei in bone marrow cells of Swiss mice *(1,39,40)*.

An ethyl acetate extract of Indian chewing tobacco induced sister chromatid exchange (SCE) in human lymphocytes and in a human lymphoblastoid cell line. In the latter system, S9 rat liver homogenate enhanced the effect. When the tobacco extract was tested in the absence of the S9 homogenate it did not induce ouabain-resistance in Chinese hamster V79 cells. The same extract, another ethyl acetate extract, and an ethanol extract of tobacco induced cell transformation in Syrian hamster embryo cells *(41,42)*.

The incidence of micronucleated oral mucosa cells in 27 Indians using khani chewing tobacco was 2.2 percent (0.8-4.9 percent). The incidence of micronuclei in exfoliated cells of nonchewers of similar ethnic backgrounds and dietary habits was 0.47 percent (0.0-0.9 percent) *(43)*.

Snuff

The residue of organic solvent extracts from a U.S. commercial snuff was dissolved in DMSO and tested for the induction of SCE's in human peripheral lymphocytes. The organic snuff extract induced significant SCE's with a 0.05 percent concentration in lymphocytes of one of three donors, with a 0.15 percent concentration in lymphocytes in two of three donors, and with a 0.5 percent concentration in lymphocytes of all three donors *(44)*.

Tobacco-Specific N-Nitrosamines (TSNA)

Of the seven TSNA so far identified in smokeless tobacco, only NNN and NNK were also tested for genotoxicity in short-term tests. In the presence of a liver microsomal preparation from Aroclor-induced rats, NNN and NNK caused dose-dependent mutations in *Salmonella typhimurium* TA100 and TA1535. Increased mutation frequencies were observed in the case of NNN at 2.5 μmol and at 5.65 μmol/plate and in the case of NNK at 1.4 μmol/plate (45-47).

NNN and NNK at 10-3 and 10-2 molar concentration each induced unscheduled DNA synthesis in freshly isolated hepatocytes from adult rats *(48)*.

Summary

Chewing tobacco and extracts from various chewing tobaccos have been tested by oral administration in mice, topical application to the oral mucosa of mice, rats, and hamsters, and by subcutaneous administration and skin application to mice. The investigations failed to demonstrate significantly increased tumor production. Short application times and low-dose exposures, however, limit the evaluation of the carcinogenicity of chewing tobacco or its extracts. Bioassays of snuff have likewise generally shown no excess cancer, although some experiments suggest that it may cause oral tumors in rats and hamsters that are infected with herpes simplex virus. Among the chemical components of snuff, the tobacco-specific nitrosamines NNN and NNK are powerful carcinogens. The doses of NNN and NNK that produce tumors in experimental animals are close to the doses estimated from lifetime exposure among human snuff dippers.

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CONCLUSIONS

1. The scientific evidence is strong that the use of smokeless tobacco can cause cancer in humans. The association between smokeless tobacco use and cancer is strongest for cancers of the oral cavity.
2. Oral cancer has been shown to occur several times more frequently among snuff dippers than among nontobacco users, and the excess risk of cancers of the cheek and gum may reach nearly fiftyfold among long-term snuff users.
3. Some investigations suggest that the use of chewing tobacco also may increase the risk of oral cancer.

4. Evidence for an association between smokeless tobacco use and cancers outside of the oral cavity in humans is sparse. Some investigations suggest that smokeless tobacco users may face increased risks of tumors of the upper aerodigestive tract, but results are currently inconclusive.
5. Experimental investigations have revealed potent carcinogens in snuff and chewing tobacco. These include nitrosamines, polycyclic aromatic hydrocarbons, and radiation-emitting polonium. The tobacco-specific nitrosamines N-nitrosonornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone have been detected in smokeless tobacco at levels 100 times higher than the regulated levels of other nitrosamines found in bacon, beer, and other foods. Animals exposed to these tobacco-specific nitrosamines, at levels approximating those thought to be accumulated during a human lifetime by daily smokeless tobacco users, have developed an excess of a variety of tumors. The nitrosamines can be metabolized by target tissues to compounds that can modify cellular genetic material.
6. Bioassays exposing animals to smokeless tobacco, however, have generally shown little or no increased tumor production, although some bioassays suggest that snuff may cause oral tumors when tested in animals that are infected with herpes simplex virus.

RESEARCH NEEDS

It has been established beyond reasonable doubt that smokeless tobacco use can increase the risk of cancer. The experimental and epidemiologic evidence is strongest for the association between oral cancer and the chronic use of snuff. Additional studies are needed to determine whether the patterns of risk differ according to the form of smokeless tobacco, including research evaluating cancer risks that are associated with chewing tobacco and dry versus moist snuff, and to quantify further the levels of risk in relation to differing levels of smokeless tobacco exposure.

The influence of smoking, alcohol, and other factors (including viral exposures) on the smokeless tobacco-associated risk of oral cancer also should be explored further with an emphasis on detecting possible interactions between these factors and smokeless tobacco.

Inhaled snuff may increase the risk of nasal carcinoma. The feasibility of initiating studies in areas where snuff sniffing is common should be ascertained, and studies should be launched to confirm and quantitate this possible relationship.

There have been few studies of smokeless tobacco and esophageal, laryngeal, and gastric cancers. These investigations have provided equivocal results, but in the aggregate, their findings raise the possibil-

ity of some increase in risk among smokeless tobacco users. Additional case-control studies of these neoplasms should be encouraged. These studies should be large enough to assess the risks that are associated with smokeless tobacco use while controlling for the potential confounding effects of smoking, alcohol, and other risk factors.

Isolated reports have associated smokeless tobacco with cancers of the cervix, pancreas, and other anatomic sites. Investigators with existing data from case-control studies of these neoplasms should be encouraged to perform analyses to determine whether associations with smokeless tobacco exist. Similarly, existing data from cohort studies with information on smokeless tobacco use should be analyzed. Reports from two relatively large cohort studies have been published only as abstracts. These should be expanded with detailed descriptions of both the methods used and the findings for various cancers and should be updated to include followup into the 1980's. Recommendations for additional studies of the role, if any, of smokeless tobacco in the etiology of cancers outside of the upper aerodigestive tract should await the results of these analyses.

On the basis of current knowledge, it can be assumed that chewing tobacco and snuff contain several unknown nitroso compounds that may be contributors to the carcinogenic potential of these products. In-depth analytical studies are needed for the identification of these unknown compounds. Furthermore, mechanisms of their *in vitro* and endogenous formation should be studied together with those of the nitroso compounds that are already known to occur in smokeless tobaccos. For the validation of the uptake of the major carcinogens by tobacco chewers and snuff dippers, markers should be measured in the target tissues and in physiological fluids. Major emphasis should be placed on the identification and assays of DNA-adducts with tobacco-specific compounds in tissues of the oral cavity.

Finally, trends over time in age-specific oral cancer incidence and mortality rates should be monitored to determine whether the increasing use of smokeless tobacco by Americans is influencing national or regional cancer patterns. Changes in the prevalence of use and in the characteristics of smokeless tobacco products should also be documented. Such monitoring will provide a base upon which future investigations of associations between smokeless tobacco and cancer can be built.

Chapter 3.

NONCANCEROUS AND PRECANCEROUS ORAL HEALTH EFFECTS ASSOCIATED WITH SMOKELESS TOBACCO USE

CONTENTS

INTRODUCTION

This chapter addresses the health effects of smokeless tobacco use on the oral tissues through a systematic review of the relevant scientific literature of animal and human studies. The major areas addressed are the effects of smokeless tobacco use on the oral soft tissues, the periodontium, and the teeth. This chapter also reviews information regarding the potential of oral tissue altered by smokeless tobacco use to transform to dysplasia and malignancy.

Within each area, except for the section on the transformation of oral soft tissues, those tissues or conditions that are suspected to be most affected by smokeless tobacco use, or that hold the greatest potential for health effects, are considered initially. Where contradictory evidence exists, these data are also presented. Studies that were judged to meet stringent selection criteria* are presented first, followed by data from less rigorous study designs and case reports.

Within the section on the transformation of oral soft tissues, the presentation of the evidence is grouped according to clinical reports, cohort studies, and case-control studies. This was done so as to be consistent with the format used in the chapter on Carcinogenesis Associated With Smokeless Tobacco Use (chapter 2). In some cases, studies referenced in this chapter are the same as those used in chapter 2. The reader should review both chapters to obtain all pertinent information contained in these studies.

Only studies from the United States and Scandinavia are included for the sections on oral leukoplakia/mucosal pathology, gingival and periodontal tissues, and salivary glands. This assures that studies dealing with similar types of smokeless tobacco are used for comparison purposes. However, the section on the transformation of oral soft tissues includes a fuller range of studies that have reviewed the histopathologic changes associated with smokeless tobacco-induced lesions. Studies investigating the histopathologic transformation of nonsmokeless tobacco-induced lesions have not been included.

A summary of selected studies that addresses study sample, methods, and observations is provided in table 1 as a ready alphabetical reference to the text. In addition, a summary of selected case reports is provided in table 2. Emphasis has been placed on the issues of prevalence of oral tissue changes, types of changes, site-specificity of changes, and the effects of dose-response.

* See Introduction, Overview, and Conclusions for discussion of criteria for causality.

TABLE 1.—Selected Study Summaries for the Noncancerous Oral Health Effects From the Use of Smokeless Tobacco

Study	Sample	Methods	Observations	Comments
Axéll, 1976			**Leukoplakia/ Mucosal Pathology**	
	• 20,333 Swedes: 51% females, 49% males. • Ages 15 years and older.	• Cross-sectional design. • Data collected on tobacco habits, medications taken, oral hygiene status, and prosthetic status. • Clinical examinations utilized diagnosis based on specific clinical criteria. • Photographic documentation of all lesions diagnosed as leukoplakia or lichen planus. • Tissue specimens taken of selected cases. • Statistical analysis conducted: t-tests, chi square tests, and, if appropriate, Fisher's exact test.	• Of 1,444 snuff users, 116 (8%) had "snuff dipper's lesion" (oral leukoplakia). • The prevalence of oral leukoplakia was 3.6% among the total population examined.	• It is not clear how many of the snuff users were also tobacco smokers. • Snuff dipper's lesion implies mucosal tissue changes at the site of snuff placement.
Greer and Poulson, 1983			**Leukoplakia/ Mucosal Pathology**	
	• 1,119 teenagers in grades 9-12. • 117 (10.5%) smokeless tobacco users: 113 males, 4 females. • Denver, Colorado.	• Cross-sectional design. • Questionnaire administered to determine years of use, frequency of use, brand of tobacco used, site of application, use of other confounding agents, and dental care history.	• A suggested association between level and duration of smokeless tobacco use and mucosal lesions (42.7% of smokeless tobacco users had oral mucosal lesions).	• An analysis of the influence of cofactors was not conducted. • No statistical analyses reported. • Examiners blind to responses on questionnaire. • No comparisons reported between users of smokeless tobacco and nonusers.