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Smokeless Tobacco and Oral Cancer: An Assessment of Evidence Derived from Laboratory Animals

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Abbreviations: BaP = benzo[*a*]pyrene; DMBA = 7; 12-dimethylbenzanthracene; HPV = human papilloma virus; HSV = herpes simplex virus; MCA = 3-methylcholanthrene; NNK = 4-(methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone; NNN = *N*-nitrosonornicotine; NQO = nitroquinoline-*N*-oxide; PAH = polyaromatic hydrocarbon; PG = propylene glycol; SEM = scanning electron microscopy; ST = smokeless tobacco; TSNAs = tobacco-specific nitrosamines.

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Historical background

The tobacco plant (*Nicotiana tabacum* and *N. rustica*) is thought to have originated on the mainland between North and South America over 7000 years ago (Voges, 1984). According to Christen *et al.* (1982). American Indians were the first to smoke, chew and snuff tobacco. The origins of these habits are lost in antiquity but some clues exist suggesting that tobacco may have afforded some relief from the rigours of life in those ancient times as well as being a pleasurable pastime. Thus, according to Heimann (1960) and Stewart (1967), the explorer Amerigo Vespucci thought that the inhabitants of a small

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island off the coast of Venezuela chewed tobacco to quench their thirst because the island was very short of water, and chewing tobacco induced profuse salivation. Chewing tobacco could assuage the effects of fatigue and hunger as well as thirst; in fact, it has been reported that an Indian could trek for 2 or 3 days with no other support than tobacco. Tobacco was also thought to have medicinal properties by the Indians and to be a good cleansing agent for teeth. Indeed, the fibrous nature of the tobacco plant may have been an effective tooth cleanser in the absence of any other suitable material.

Chewing tobacco

According to Gottsegen (1940) and Brooks (1952), tobacco chewing became popular in eastern USA during the first half of the 19th century and the custom spread to Europe. Tobacco chewing was found to be a good substitute for smoking by those who could not smoke or who were prevented from doing so. For example, smoking was a fire hazard on board wooden ships and was therefore banned, so sailors turned to chewing tobacco (Brooks, 1952). The belief in the beneficial effect of tobacco chewing against illness was also prevalent in Europe and no doubt helped to popularize the product.

There was an undesirable side to the practice of chewing tobacco. Some users expectorated the chewed cud indiscriminately, thus creating a nuisance in public places, while the advent of the germ theory of disease led to the fear that such practices may present a serious hazard to health by disseminating germs, in particular tuberculosis (IARC, 1985). Thus, the practice of chewing tobacco became less popular towards the end of the 19th century and the beginning of the 20th century but is still widespread today.

Inhaled powdered tobacco

The practice of inhaling powdered tobacco is thought to have originated among the Indians of Brazil. They ground the tobacco leaves to a powder in a rosewood cup and inhaled the powdered leaves via ornate bone tubes (Curtis, 1935). According to Christen *et al.* (1982) and Stewart (1967), the practice of inhaling powdered tobacco leaves was widespread among the inhabitants of Mexico and the Caribbean Islands, notably in Haiti and the lesser Antilles. Snuff was introduced into Europe by members of the second expedition of Columbus to the New World, and at first it was thought to possess medicinal properties, a belief presumably borrowed from the Indians. The inhalation of snuff gradually spread throughout the then known world and became a socially acceptable practice, particularly in Europe. Snuff use reached a peak in England during the reign of Queen Anne (1702–1714). It continued to be popular for several decades afterwards and, according to records, it was used by several prominent people such as Lord Nelson, the Duke of Wellington, Alexander Pope and Samuel Johnson (Harrison, 1964).

A note on current practices

Chewing tobacco and snuff are currently called smokeless tobacco (ST). Snuff consists of tobacco that has been cured and then finely ground to produce dry snuff (< 10% moisture) called "Scotch", moist snuff (up to 50% moisture) or fine-cut tobacco, the latter being generally considered a form of moist snuff.

The customary use of snuff involves "snuff dipping". The practice consists of taking a small amount of snuff between the gingiva and either the lip or the buccal mucosa. It then can be left for a few minutes and cleared by expectoration or by swallowing, or left for much longer periods; some users retain snuff in this position for several hours. Snuff dippers usually expectorate saliva mixed with tobacco extract.

Chewing tobacco can be obtained as plug, loose-leaf and twist or roll tobacco. Plug tobacco is made from tobacco leaves that are wrapped in fine tobacco and made into flat bars or rolls. Loose-leaf tobacco is formed from fermented cigar leaf tobacco which is not compressed. Twist or roll tobacco is composed of cured, flavoured leaves that have been twisted into strands and dried. Chewing tobacco is held in the mouth where it can be chewed intermittently for several hours. The saliva mixed with tobacco extract is usually expectorated.

The use of ST varies considerably from state to state in the United States and from country to country in western Europe and the Far East (Hunter *et al.*, 1986; Marty *et al.*, 1986). In the US the custom of using ST is particularly prevalent among the native Indian population (Cullen *et al.*, 1986). The consumption of ST in the US is reported to have tripled between 1972 and 1991, when it is estimated to have involved 5.3 million adults (Anon, 1993). These ST users were predominantly men and represented 5.6% of the adult male population. The greatest usage was in native American and non-Hispanic white males and the usage was inversely correlated with the number of years in education. In Sweden, about 15% of adult males use moist snuff (Lewin *et al.*, 1994).

Recently, tobacco enclosed in fabric material of the same type as that used in tea bags has been offered to users. About 1 gram of tobacco is contained in a bag approximately 2 inches square. The bag is placed between the gingiva and the cheek and is either left in place or worked intermittently. This method of presenting tobacco allows extraction of nicotine by saliva and improves oral hygiene in tobacco users by retaining the fibrous residues in the bag.

Over the years there have been many publications in the scientific literature dealing with ST and oral cancer. A number of epidemiological studies have

been conducted to ascertain whether ST is associated statistically with this disease. These were evaluated by a working group from IARC, who concluded that "there is sufficient evidence that oral use of snuffs, of the type commonly used in North America and western Europe, is carcinogenic to humans. There is limited evidence that chewing tobacco of the type commonly used in these areas is carcinogenic" (IARC, 1985). This problem was studied carefully and in detail in subsequent investigations (Lewin *et al.*, 1988; Mashberg *et al.*, 1993; Muscat *et al.*, 1996; Sterling *et al.*, 1993). The findings did not support the conclusions made by IARC (1985) and so the question regarding the carcinogenicity of snuff is still unanswered. In addition, a large amount of experimental work has been carried out to investigate the possible carcinogenicity of ST in laboratory animals. Various models have been employed in this endeavour. The purpose of the current review is to give an account of the experimental research carried out with ST products used in the US and western Europe, which consists essentially of ST. The Asian tobacco products are not included because they are substantially different from those used in the US or western Europe.

Studies on whole smokeless tobacco

Studies in the oral cavity

Hamster—cheek pouch. The hamster possesses two pouches, one on either side of the mouth, which open into the oral cavity and lie underneath the muscles of the cheek, hence the name "cheek pouch". The openings of the pouches lie in the anterior part of the oral cavity and are associated with small salivary glands, which produce both serous and mucous secretions. The pouches extend backwards along the oral cavity but do not reach as far as the oropharyngeal junction. Histologically, the lining of the buccal cavity consists of keratinizing squamous epithelium (Emminger and Mohr, 1981).

Functionally, the cheek pouch stores half-chewed food, which is pressed out as needed. Tobacco and snuff remain *in situ* for several hours when introduced into the pouch and are periodically extruded in small amounts for chewing (Shklar *et al.*, 1985). Thus, the hamster cheek pouch appears to be suitable for the study of prolonged exposure of the oral mucosa to tobacco products.

One of the earliest studies of the possible carcinogenicity of ST was carried out in this model by Peacock and colleagues (Peacock and Brawley, 1959; Peacock *et al.*, 1960). The authors questioned the relevance of the tumours obtained in earlier studies, involving repeated application of distillates of cigarette tobacco on mouse skin, for assessing the carcinogenicity of unburned tobacco. They selected the hamster cheek pouch because it appeared to simulate snuff dipping in man. In addition, the absorption of compounds from the pouch into the systemic circulation was slow, allowing prolonged exposure of the pouch epithelium to the test substance. For example, implantation of 40 mg strychnine (eight times the minimum lethal dose for hamsters) into the pouch did not result in the rapid deaths which would otherwise have occurred from direct oral dosing.

In their experiment, Peacock and colleagues (Peacock and Brawley, 1959; Peacock *et al.*, 1960) used 124 hamsters (sex unspecified). After dissecting out the cheek pouch and widening the oral opening of the pouch, the authors inserted 10 cm^3 snuff or 2 cm^3 chewing tobacco plug and then ligated the sac and returned it to the original position underneath the cheek muscle. Sixty hamsters received the snuff and the other 64 received chewing tobacco. The test material was implanted in the left pouch while the materials used as controls (sand or some bland material) were implanted in the right pouch. A mild chronic infection, which appeared to be self-limiting, occurred in a few pouches but did not progress beyond the third week. The experiment lasted 30 months. In the group that received snuff, 19 died within the first 12 months, 11 within the next 12 months and the 10 survivors within the next six months. In the group that received chewing tobacco, 43 had died within the first 12 months, 13 within the next 12 months, and the remaining eight within the next 6 months. No tumours were found in the mucous membrane of the pouch or oral cavity in either of the groups.

Dunham and Herrold (1962) investigated the possible carcinogenicity of snuff in a group of 35 hamsters. The snuff was incorporated into a beeswax pellet and inserted through an incision into the isolated pouch. The pellet contained 20% snuff and was left in place until the end of the experiment at 2 years. An inflammatory reaction of the pouch was observed in two animals. No tumours were observed in the pouch or oral cavity.

The laboratory carcinogens 7,12-dimethylbenzanthracene (DMBA) or 3-methylcholanthrene (MCA) served as positive controls. They were incorporated into beeswax and inserted into the cheek pouch of 71 hamsters. Serial killings in these two groups during the first 5 months revealed the development of acute inflammation, ulceration and necrosis of the mucous membrane of the cheek pouch. Twenty of 23 animals exposed to DMBA or MCA developed carcinomas and sarcomas after six months.

In later experiments by Dunham *et al.* (1966), snuff was tested in a group of seven hamsters. At the termination of the experiment, their average age was 99 weeks. Snuff (50 mg) was inserted by a child-size nasal speculum once daily on 5 consecutive days each week throughout the experiment except during the first 2 weeks, when the amount

administered was 250 mg/insertion. Starch was inserted into a group of four animals and served as a negative control. There were no reactive changes or tumours in the pouches of hamsters treated with snuff or with starch powder. In another experiment (Dunham *et al.*, 1975), chow containing 2.5% of snuff was fed to a group of two male and two female hamsters daily for 5 days a week for up to 2 years. No tumours were observed.

Hamster oral mucosa. Homburger (1971) investigated the possible carcinogenic properties of ST on the oral mucosa and cheek pouch of the hamster. He immobilized the animals' heads by a stanchion for 30 minutes each day, which allowed snuff to be applied with an automatic cartridge filler to the gingivolingual area, including the upper part of the buccal pouch. One experiment was terminated at 8 months according to the author's table (30 weeks according to the text) because of high mortality. In another experiment, groups of 35 males and 25 females were allocated to one of the following treatments: snuff, cotton (dry, control), benzo[*a*]pyrene (BaP) and DMBA. The snuff was applied neat, while the carcinogens were applied on absorbent cotton as 0.2 ml of a 0.5% solution in acetone or peanut oil. Daily exposure continued for 1 year except for DMBA, which was continued for only 30 weeks because the animals' health deteriorated. The experiment was terminated after 1 year. One male and two females died from the untreated controls. In contrast, the number of survivors for males and females in the group treated with dry cotton were 15 and 14, in the snuff-treated group 15 and nine, in the BaP-treated group 17 and seven, and in the DMBA-treated group 10 and four. From the beginning of the experiment, hamsters exposed to snuff were noted to cease to struggle and some actually went to sleep, presumably because of the calming effect of the tobacco. In contrast, animals exposed to cotton or to carcinogens continued to struggle during the 30 minutes of restraint. The authors attributed the high mortality to cervical dislocation brought about by the struggle against restraint.

The epithelium of the lip, of the oral cavity, and of the cheek pouch of the animals exposed to snuff showed visually only minor changes compared with controls. Focal epidermal hyperplasia was observed microscopically in six snuff-exposed and two cotton-exposed animals (sex unspecified). One benign tumour (papilloma) was found in each of the snuff-exposed and cotton control groups.

A marked hyperplasia and metaplasia (change in cell morphology) occurred in the majority of animals treated with BaP. Similar but less severe lesions occurred in hamsters treated with DMBA. Ten squamous cell carcinomas, three in the pouch, five in the skin and two in the mouth, developed in the DMBA-exposed group, whereas only three tumours were observed in the group of animals treated with BaP, one each in skin, oral and pouch mucosa. (Homburger, 1971).

The lesions produced by snuff insertion into the right buccal cavity of hamsters were studied in detail by Ashrafi *et al.* (1992) on a group of 24 Syrian golden hamsters. A group of eight hamsters served as controls. Approximately 2 g of commercially available American manufactured moist snuff were placed into the blind end of the right buccal pouch once a day on 5 days a week for 24 months. The left pouch was untreated but it was gently touched by a glass rod every time snuff was inserted into the right buccal cavity. The animals were allowed free access to laboratory diet and drinking water. Seven out of eight untreated controls and 22 out of 24 experimental animals survived until the end of the experiment at which time they were sacrificed. No tumours were reported in this study. The pouch epithelium of the untreated animals showed no changes from normal. In the treated animals, the cheek pouch became hyperplastic and hyperkeratotic with a prominent granular layer. By transmission electron microscopy the spinous cells were separated from one another more than normal and the spaces were filled with microvilli projecting from the plasma membrane. An increased number of mitochondria and of rough endoplasmic reticulum was also observed. By scanning electron microscopy (SEM) the changes in the epidermis consisted of the development of an irregular arrangement of the micro ridges and a disappearance of the normal honeycomb pattern (Ashrafi *et al.*, 1992).

Hamster—short-term studies. Shklar *et al.* (1985) introduced 70 mg finely powdered snuff or 50–100 mg coarser tobacco daily for 20 weeks into the cheek pouch of 20 male hamsters. The experiment was terminated at 20 weeks. While no significant pathological changes were observed in these animals, there was a slight diminution of mitotic activity and an increase in Langerhan's cells.

Similarly, the application of 2 g commercially available American snuff in the blind end of the right buccal pouch of a group of eight male hamsters daily, 5 days a week for 6 months (terminated at 6 months) resulted in hyperplasia of the buccal cavity epithelium. A roughening of the surface was observed by SEM, while whitish patches were observed visually (Worawongvasu *et al.*, 1991). No focal proliferative lesions or tumours were found.

Comment

As no tumours or dysplastic lesions were observed in the hamster oral cavity or cheek pouch in the experiments outlined above, it would appear that ST is not carcinogenic in the model systems reported in this section. Furthermore, ST, despite its particulate nature, elicited a very mild reactive lesion at the site of application comparable to that

produced by cotton or starch used as controls. This mild irritant response is in strong contrast to the marked reactive lesion produced by the insertion of the same materials in the rat artificial lip canal, which is discussed in the next section.

Rat—artificial lip canal. Hirsch and Thilander (1981) developed a rat model designed to simulate human "dipping" by facilitating contact between snuff and saliva. Using microsurgical techniques, they created an artificial canal in the lower lip of a young adult rat, which was open at both ends and was lined internally by a mucosal epithelium and externally by skin from the lip. Tobacco or similar solid products could then be inserted and left in place or replaced as required. The operation initially caused a marked inflammatory reaction, but in one animal, killed 14 days later, the reaction had almost completely subsided. At this time the lumen of the canal was covered by keratinized squamous epithelium, of the type seen in the buccal cavity. It was acanthotic (5–10 cell layers) with rete pegs projecting into the submucosal layer.

The authors do not state the interval of time allowed for healing between the operation and first insertion into the lip canal. As far as one could see, the tissue reaction at the site of surgery was allowed to heal before commencing an experiment. The test material was injected into the artificial lip canal by a plastic syringe until the excess was pressed out through the buccal opening, ensuring complete filling of the canal. The authors found that this model could accommodate approximately 0.25 g powdered snuff, which corresponds to a mean dose of 1 g/kg body weight (approx. five times the amount a human might use). The inserted material was retained for 5 to 8 hours and was accompanied by "hypersalivation" and an increase of blood nicotine from 13 ng/ml (in one control) to 83 and 250 ng/ml (two animals). After a twice-daily application of 0.2–0.4 g powdered snuff for 9 months, the epithelium of the canal was mildly to moderately hyperplastic and the adjacent connective tissue exhibited an inflammatory reaction, which varied in degree from mild to severe (Hirsch and Thilander, 1981). This model was utilized by Hirsch and Johansson (1983) to study the effect of long-term application of snuff on the oral mucosa of the rat. These authors inserted 0.2 g standard snuff or snuff made more alkaline than normal by the addition of sodium carbonate into an artificially created lip canal twice daily for 9 to 22 months. Twenty one male and 21 female rats received the standard snuff, groups of six to eight of each set were killed at 9 and 12 months. The remaining rats were healthy up to 18 months, after which there was a decline so that they were killed between 18 and 22 months. The 15 rats in the control group (which had the lip canal but were not treated with snuff or other material) were killed in three groups at 9, 12 and 18 months.

After 9 to 12 months of treatment with standard snuff, the squamous epithelium of the canal exhibited a generalized mild to moderate hyperplasia but foci of severe hyperplasia also occurred. A disturbed polarity of epithelial cells was observed in some of the foci. The underlying connective tissue exhibited a mild to moderate inflammatory reaction at 9 months. The reactive lesions in the epithelium and submucosa showed virtually no further changes as the experiment progressed but the fibrosis, which was observed in the submucosa at 9 months, became more prominent later on.

The histological picture produced by alkaline snuff in the tissues of the lip canal was similar to that produced by standard snuff, while only a minimal to mild epithelial hyperplasia was observed in untreated controls. A single invasive squamous cell carcinoma was found in the oral cavity of a rat that had been treated with alkaline snuff for 8.5 months. According to the authors, the tumour might have been either spontaneous in origin or produced by treatment. The exact site of the carcinoma within the oral cavity was not specified. Rats that had been exposed to alkaline snuff for 10–22 months had a marked papillary hyperplasia of the forestomach, an organ not found in humans (Hirsch and Johansson, 1983).

The appearance of some dysplastic foci in the hyperplastic epithelium of the lip canal in animals repeatedly treated with snuff prompted a follow-up investigation to find out whether these dysplastic foci would be followed by the development of tumours if the animals were allowed a treatment-free period before terminating the experiment (Hirsch *et al.*, 1986). Commercially available snuff (0.2 g) was inserted twice daily into the artificial lip canal of 30 male Sprague–Dawley rats for 13 months. Ten rats were killed at the end of the treatment period, another 10 were killed one month later and the last group was killed 4 months after cessation of treatment. Ten control rats were subjected to the same surgical procedure but were left untreated and were killed at month 13 of the study. In all groups, tissues were removed from the gingiva, tongue and buccal mucosa for examination. Histologically, in sections from controls, a slight hyperkeratosis and acanthosis was present in the mucosal epithelium of the lip canal but the rete pegs were not prominent and the inflammatory reaction in the subepithelial tissue was mild or absent. In the 10 test animals killed at the same time (13 weeks), there was a mild to moderate squamous hyperplasia and hyperkeratosis. Acanthosis was slight to moderate with marked development of rete pegs and focal atypia in the basal layer. The inflammatory reaction in the submucosal connective tissue varied from slight to severe but fibrosis was prominent in all treated animals. The reaction of the mucosal epithelium and the inflammatory infiltrate was less prominent after a 1- or 4-month treat-

ment-free period but the fibrosis remained unaltered.

Ulceration and a moderate to severe hyperkeratosis and hyperplasia were observed in the gingival epithelium of treated rats killed at 13 and 14 months, but the lesions were much less severe in rats killed after a treatment-free period of 4 months. The epithelium of the tongue and buccal mucosa in treated rats was slight to moderately hyperplastic at all observation time points.

The artificial lip canal was subsequently employed to explore further the possible carcinogenicity of snuff in the oral cavity of rats (Hecht *et al.* 1986; Table 1). Snuff (50 mg), obtained commercially, was inserted into the lip canal from the oral cavity side using the cap of a JELCO catheter placement unit and a steel plunger. Insertion was made five times weekly for up to 116 weeks in a group of 32 male F344 rats. In the second group, the solid remaining after extracting snuff with water and filtering (extracted snuff) was air-dried and inserted into the lip canal of 21 rats daily in the same way and for the same number of weeks. In the third group, the filtrate was freeze-dried and then brought to the same consistency of moist snuff by the addition of water. It was then mixed with commercial snuff, and 50 mg of this mixture (designated enriched snuff) was inserted once daily in the lip for 116 weeks in a group of 32 rats. Ten animals that had been subjected to the surgical construction of the lip canal were left untreated and served as controls. No tumours were observed in the oral cavity of the controls. In the group treated with commercial snuff, two tumours, one of which was malignant, developed in the epithelium of the lip canal and there was one papilloma of the hard palate. In the group treated with extracted snuff, one rat had a papilloma of the tongue and another a papilloma of the hard palate. One animal in the group treated with enriched snuff developed a papilloma of the floor of the mouth. No sarcomas were reported. The authors stated that the incidence of tumours in snuff-treated rats was not statistically significant compared with controls, although the incidence was higher than in some other studies with F344 rats (Hecht *et al.*, 1986). Non-specific lesions of the oral cavity including hyperkeratosis, acanthosis, chronic or acute inflammation and granulomas were observed.

Long-term application of ST to the lip and oral cavity of rats results in the development of a few tumours at the site of application. In order to investigate the mechanism by which the tumours originate, snuff, cotton wool or surgery were applied to groups of 20 rats and the rate of cell proliferation measured by a technique involving incorporation of bromodeoxyuridine (BrdU). The labelling index of the epithelium of the lip was significantly greater in the group of rats treated with snuff compared with that subjected only to surgery at 6 and 12 weeks. At 6 weeks, the labelling index of the oesophagus from animals treated with snuff was greater than that of rats which had surgery, and at 12 weeks it was greater than that from animals subjected to cotton wool treatment and surgery (Saidi and Johansson, 1991). It would appear that hyperplasia plays an important role in the development of tumours at the site of snuff insertion in this model.

Comment

The artificial lip canal technique has some advantages in the investigation of oral carcinogenesis in short-term tests. The canal is readily accessible for the insertion of test material and saliva enters the canal from the oral cavity in copious amounts, facilitating the elution of solutes (Hirsch and Thilander, 1981). There is, however, a major drawback in the model that raises substantial doubts about the validity of the results obtained in long-term studies. This lies in the fact that the surgical procedure itself creates a marked inflammatory response. Although this response subsides within 2–3 weeks of the operation, it leaves a mildly hyperplastic epithelium with scar tissue formation which persists for up to 13 months (Hirsch and Thilander, 1981). In the study by Hecht *et al.* (1986) the insertion of snuff is bound to have caused mechanical injury, which in turn would have had the effect of reactivating the inflammatory process resulting in marked epithelial hyperplasia and a progressive increase in scar tissue, both of which would have persisted as long as the treatment continued (Hirsch and Johansson, 1983). It is well known that proliferative reactions of this sort in epithelial or connective tissues are prone to lead to cancer induction in laboratory animals even when no carcinogens are applied (Anderson, 1991; Clayson *et al.*, 1991;

Table 1. Tumour incidence[a] in male F344 rats[b] after the application of snuff in the lip canal (from Hecht *et al.*, 1986)

Group	No. of rats	Lip canal	Tongue	Hard palate	Floor of mouth	Nasal cavity	Stomach
Control	19	0	0	0	0	0	1
Snuff	32	1 – (1)	0	1	0	0	1
Extracted snuff	21	0	1	1	0	0	1
Enriched snuff	32	0	0	0	1	(1)	2

[a] Numbers in parentheses indicate malignant tumours, all others were papillomas.
[b] Surviving to 116 weeks.

Table 2. Incidence of proliferative lesions in male Sprague–Dawley rats (28 or 29 per group) after the application of NQO to the hard palate and snuff to lip canal (from Johansson *et al.*, 1989)

	Tumours					Squamous cell hyperplasia				
	I	II	III	IV	V	I	II	III	IV	V
Lip and lip canal	1 + (1)	—	—	(1)		24	6	4	25	10
Hard palate	1 + (2)		(2)	(4)		18	7	7	14	2
Tongue			2 + (2)	1 + (1)						
Nasal cavity	1 + (1)	—	—	(1)		—	—	—		
Oesophagus			(1)	—	—	—	—	—	—	—
Forestomach	(1)	—	(2)	(2)	—	18	4	6	18	1
Lip (sarcomas)	2	—	—	3	—	—	—	—	—	—

Numbers in parentheses indicate benign tumours.
Group I = Snuff insertion for 104 weeks.
Group II = Painting hard palate with PG for 4 weeks.
Group III = Hard palate painted with NQO in PG for 4 weeks.
Group IV = As in group III but with snuff inserted in lip canal for a further 104 weeks.
Group V = Insertion of saline in cotton wool for 104 weeks.

Ingram and Grasso, 1991; Poynter and Selway, 1991).

The number of epithelial tumours in the lip canal studies is low and most of them are benign. This picture is well in keeping with the low incidence of tumours produced by non-carcinogenic irritant materials in mouse skin or bladder epithelium in mice and rats. Furthermore, the fact that no tumours were found in an experiment in which animals were treated twice daily for 13 months and terminated at 14 months, would suggest that the tumours observed in a subsequent investigation by Hecht *et al.* (1986) may have originated after 13–14 months of treatment. A latent period of this duration is in keeping with that observed in the experimental induction of tumours by agents that induce persistent tissue injury (Grasso *et al.*, 1991).

In addition, the experiments with snuff in the lip canal are lacking in adequate controls. For example, no biologically inert material (such as cotton wool) was inserted in the control animals. Instead, the control group was not subject to any further manipulation, while the test animals were subjected to repeated application of native or modified snuff, a procedure which must involve considerable trauma judging by the severe epithelial and connective tissue reaction that it provokes (Hirsch and Johansson, 1983)

Thus, the investigations using the artificial lip canal model have not shown that snuff possesses any carcinogenic activity. In our view, the marked reactive lesion seen histologically in the lip canal following repeated insertion of snuff is sufficient to account for the type and number of tumours observed.

Promotion studies on smokeless tobacco

The concept of an agent incapable of inducing tumours in its own right but capable of doing so if applied to tissue cells already "primed" or "transformed" by a carcinogen, arises from the early studies on the "two-stage" mechanism of cancer production on mouse skin by carcinogenic polycyclic aromatic hydrocarbons (PAH).

The two-stage process is often referred to as the "initiation/promotion" model of carcinogenesis. In this model, a single or a few (e.g. 4–10) applications of a subcarcinogenic dose of a carcinogen (e.g. PAH) to the same site on mouse skin, would not give rise to tumours. But the application of a promoter (e.g. croton oil) for up to 10–13 weeks at the same time as the carcinogen, resulted in the production of papillomas, some of which progressed into carcinomas. Thus the carcinogen "initiated" the cells, but to complete the neoplastic transformation, the "promoter" was necessary (O'Connell *et al.*, 1987; Roe, 1956a,b; Salaman and Roe, 1956). Unfortunately, no croton oil "controls" were included in these early experiments so that there was no knowledge of the effect of the promoter *on its own* on tumour induction. Salaman and Roe (1956) investigated this problem and found that the application of promoter alone was followed by tumour development. This finding was confirmed by other workers with promoters other than croton oil so that the model was now regarded as unreliable for assessing human risk (Boutwell, 1989; McKee *et al.*, 1989).

Nevertheless, the system was employed by Johansson *et al.* (1989) to discover whether snuff possesses any possible "promoting" activity (Table 2). A group of 150 SD rats had an artificial lip canal created surgically in the lower lip and were then divided into five groups. Group I rats received 50 mg snuff, 5 days a week for 104 weeks. Group II rats had propylene glycol (PG) painted on the hard palate three times weekly for 4 weeks only (vehicle control). Group III rats had nitroquinoline-*N*-oxide (NQO), made up as a 0.5% solution in PG, painted three times weekly for 4 weeks on the hard palate of rats and then left undisturbed for the rest of the experiment ("initiating" treatment). Group IV rats had the same treatment as group III rats, followed by snuff five times weekly as in group I ("promoting" treatment). Group V rats received cotton

Table 3. Incidence of squamous cell tumours in rats treated with HSV-1 or NQO and snuff in the lip canal (from Larsson *et al.*, 1989)

		Carcinoma				Papilloma		
Group	No. of rats	Ear duct	Lip	Oral cavity	Nose	Forestomach	Lip	Forestomach
I	12	1	1			—	—	—
II	13	—		1	1			—
III	15	1	—	—			—	—
IV	12		2	1	—	—	—	1
V	12	—	—	2		1	1	—
VI	8	—	—	—	—	—	—	

All animals had an artificial lip canal.
Group I = HSV-1 inoculation.
Group II = Snuff.
Group III = HSV-1 inoculation + snuff.
Group IV = NQO.
Group V = NQO + snuff.
Group VI = control.

soaked in physiological saline 5 days weekly for 104 weeks when the experiment was terminated. There was a low incidence of epithelial tumours in all treated groups but none in control groups II or V. Most epithelial tumours occurred mainly on the hard palate and tongue. The authors concluded that there was no evidence of any promoting effect by snuff because the tumour incidence in the group treated with NQO followed by snuff was the same as that treated with NQO alone. In addition, two sarcomas appeared in group I and three in group IV.

The possibility that snuff could promote the carcinogenicity of NQO was studied in another experiment in the artificial lip canal model by Larsson *et al.* (1989; Table 3). An artificial lip canal was constructed surgically in two groups of 12 rats each. After healing, the lip canal was exposed to a solution of NQO in 0.5% propyl gallate daily for 4 weeks (group IV) and was left undisturbed. The other group was similarly treated and then had 200 mg Swedish snuff inserted twice daily 5 days a week for 30 months (group V). A group of eight animals had surgery only and served as controls (group VI), and another group (group II) had surgery and snuff as in group V, but no NQO. The results show a low incidence of carcinomas in and near the lip canal in the groups treated with NQO alone or with NQO followed by repeated snuff. There was no significant difference between these incidences and therefore this is further evidence that snuff does not promote the carcinogenicity of NQO.

Johansson *et al.* (1991; Table 4) also used the artificial lip canal model to explore the possible carcinogenicity of snuff and its ability to promote tumour formation following initiation by either NQO or DMBA. In this study 330 male Sprague–Dawley rats had an artificial canal constructed surgically in the lower lip. Forty of these rats (group I) were treated by the insertion of a cotton pellet soaked in a 0.1% solution of DMBA in mineral oil into the lip canal, three times weekly for 4 weeks, and thereafter they received a cotton pellet soaked in physiological saline, once a day, 5 days per week for 104 weeks. Another group of 40 rats was treated in the same way with DMBA but then received 50 mg snuff twice daily, 5 days per week, for 104 weeks (group II). The next group of 38 rats (group III) received only snuff, twice a day 5 days per week, for 104 weeks. Group IV consisted of 40 rats treated with cotton pellets dipped in an 0.5% solution of NQO in PG three times weekly for 4 weeks and then with a cotton pellet dipped in PG twice a day for 5 days a week for 104 weeks. Group V (38 rats) was treated with NQO as in group IV and then with snuff as in group III.

Table 4. Tumour incidence in male Sprague–Dawley rats (38–42 per treated group with 30 controls) after the application to the lip canal of NQO or DMBA followed by snuff (from Johansson *et al.*, 1991)

	Sarcomas						Squamous cell tumours					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI
Lip canal	—	9	10	1	25	1	—	1	(2)	1 + (1)	1 + (2)	—
Hard palate	—	—	—	1	—	—	—	2	3	6	5	—
Buccal mucosa		—		—	—	—		—	—	1	—	—
Nasal cavity	—	—	—	—	—	—	1		—	1	—	
Forestomach	—				—			2	—	—	1	—

Numbers in parentheses indicate benign tumours.
Group I = DMBA plus cotton pellet.
Group II = DMBA plus snuff.
Group III = Snuff.
Group IV = NQO plus cotton pellet.
Group V = NQO plus snuff.
Group VI = Saline plus cotton pellet.

Controls consisted of 30 rats treated with cotton pellets dipped in physiological saline.

Sarcomas occurred in the lip canal in all groups except the one treated with DMBA only (group I). The highest incidence occurred in the group treated with NQO followed by snuff. A number of small squamous cell tumours—mainly malignant—occurred in groups II, III, IV, and V (Table 4). The authors concluded that there was no significant difference among these groups and that no promotional effect was observed. The incidence of tumours outside the head, neck, and gastrointestinal tract was higher in the two groups treated with DMBA than in other groups.

Comment

In the studies reviewed in this section, epithelial tumours occurred in various treatment groups including those groups treated only with snuff. These tumours were found not only in the lip canal but also on the tongue and hard palate. The incidence is at or below the level of statistical significance if the tumour incidence is analysed per anatomical region against negative controls. It achieves statistical significance in the experiment by Johansson *et al.* (1989), if tumours from the hard palate, lip canal, forestomach, and nasal cavity in group I are added together and the combined incidence compared with the zero incidence in controls (group II). According to McConnell *et al.* (1986), it is permissible to group together tumours of the same histogenetic origin but not if the cell of origin is different. Thus, the inclusion of nasal tumours in the group invalidates the statistical analysis used by Johansson *et al.* (1989).

The number of epithelial tumours induced by snuff insertion in rats pretreated with DMBA is low and of the same order as in rats treated by snuff alone, so that snuff did not act as a promoter in this part of the study. Similarly snuff did not "promote" tumours in animals pretreated with NQO, since there was no difference in the epithelial tumour incidence between groups treated with NQO alone and those treated with NQO + snuff in all three experiments. The high incidence of epithelial tumours in the experiment reported by Johansson *et al.* (1991) is probably due to the relatively high dose of NQO given (five and eight times the amount of NQO used in the other studies). The forestomach lesions seen in the studies reviewed in this section included a low incidence of squamous cell carcinomas. They probably reflect the irritant properties of the high doses of snuff ingested and are not indicative of carcinogenic activity. It has been shown that forestomach tumours in rodents are caused by a number of substances that are not generally regarded as carcinogens (e.g. *d*-limonene, butylated hydroxyanisole) but which produce sustained high levels of cellular proliferation in the rodent forestomach (Clayson *et al.*, 1991).

Connective tissue tumours (sarcomas) developed almost entirely around the lip canal. A previous study of the lesions in the connective tissue surrounding the lip canal induced by snuff insertions revealed an active and progressive fibrosis which persisted even when the treatment was stopped (Hirsch *et al.*, 1986). Reactions of this sort are known to be particularly prone to lead to sarcoma production even in the absence of chemical carcinogens (Grasso *et al.*, 1991).

The relatively high incidence of sarcomas in the study by Johansson *et al.* (1991) is probably due to a severe connective tissue response to trauma occurring in tissues pretreated with NQO—a "potent intraoral carcinogen". Fibroblastic proliferation, which is at the heart of this connective tissue reaction, acts as a strong stimulus for tumour production or tumour promotion in the same way as hyperplasia in epithelial tissues (Grasso *et al.*, 1991). The frequent and prolonged use of an instrument such as a spatula would inflict sufficient physical trauma to account for the sarcomas observed.

Thus, in these studies, the severe mechanical trauma induced by the frequent cleaning and filling of the lip canal can account both for the epithelial and connective tissue tumours in the snuff-exposed animals. There is no indication that snuff promotes epithelial tumour formation after initiation by DMBA or NQO, while the evidence is consistent with the hypothesis that snuff may promote the formation of sarcomas after NQO initiation. It is probable that the tumours in the snuff-exposed rats were instead "promoted" by the reactive lesions induced by physical trauma.

Dietary carcinogenicity studies on smokeless tobacco

In order to provide further information on the carcinogenicity of ST, a diet containing dried snuff was fed to 40 male Wistar rats for 18 months and to 34 and 16 mice of the DBA and C57 Bl strain, respectively, for 15 months (DiPaolo, 1962). The dietary concentration of the dried snuff was approximately 5% for the rats and 25% for the mice at the beginning of the study, falling by stages to 5% by the end of the study. The snuff-treated groups had a statistically significant reduction of body weight. Survival was comparable to controls. Histopathology was performed on tissues that were grossly abnormal. The report on these stated that there were "few pathological changes". In the snuff-treated groups one rat had a kidney sarcoma and one rat and three mice had leukaemia. No malignancies were reported in the controls although the authors comment that tumours occur spontaneously in these animals. No malignancies occurred in the oral cavity or upper alimentary tract in the snuff-treated test groups.

Table 5. Tumour incidence in hamsters (50 of each strain per dose group) dosed orally (from Homburger *et al.*, 1976)

	Cellulose[a]	Snuff[a]	MCA[b] (5 mg/dose)	Snuff[a] + MCA (0.5 mg/dose)	Cellulose[a] + MCA (0.5 mg/dose)
Forestomach	4 + (3)[c]	1 + (7)	20 + (50)	2 + (1)	7 + (6)
Glandular stomach	0	0	19	8	6
Small intestine	0	0	8	0	0
Large intestine	1	1	51 + (1)	1	2

[a]20% in diet.
[b]MCA = methylcholanthrene.

Homburger *et al.* (1976), having previously obtained negative results using the hamster cheek pouch, turned to the dietary route for further investigation (Table 5). According to the authors' text 500 male hamsters were used in this study, 250 of the BIO 15.16 strain and 250 of the BIO 87.30 strain. However, according to the table of neoplasms in the paper, both male and female hamsters were used. The hamsters of each strain were divided into five groups. The first and second groups were given a diet containing 20% snuff or 20% cellulose, the third group received 50 doses of 5 mg methylcholanthrene (MCA) by gavage (frequency not stated). The fourth and fifth group received a low dose of MCA (0.5 mg × 50 doses) by gavage, as well as a diet containing either 20% cellulose or 20% snuff.

Food consumption was reduced in all animals receiving the diet containing snuff but the difference was not statistically significant. The body weight of hamsters of the BIO 87.20 strain maintained on 20% snuff was significantly less than those maintained on the same regimen and also given MCA by gavage. The body weights of the BIO 15.16 hamsters dosed with snuff was only slightly reduced.

The experiment continued for 2 years, when it was terminated. Twenty-two out of the total of 100 animals treated with a high dose of MCA had died during the first year, but, of the remaining animals, only 10 died in the second year. Cotinine was detected in the serum of animals receiving snuff.

Only forestomach tumours were observed in animals fed snuff or cellulose. The incidence was comparable, indicating that the commercial brand of snuff used in this study was not carcinogenic. As mentioned earlier, these tumours are not indicative of a carcinogenic risk for man. As expected, MCA, a potent carcinogen, produced malignant tumours in various organs.

Comment

This study by Homburger *et al.* (1976) is valuable because it lasted for 2 years and was carried out on adequate groups of animals with a good survival record. Furthermore, the dietary concentration, in most groups, was much higher than that normally used for non-toxic materials (5%) in these types of experiments, suggesting that the dose level was sufficiently high to reveal any potential carcinogenicity.

Studies on extracts of smokeless tobacco

Topical application

Processed tobacco contains a number of nitroso compounds of both the low molecular weight (volatile) and the higher molecular weight (non-volatile) type, derived by the interaction of nitrate/nitrite and amines found in tobacco (Tricker and Preussmann, 1991). The volatile nitrosamines are of the type found in several foods such as fish, cured meat and cheese (MAFF, 1992) and are present in relatively small amounts in tobacco. Therefore, attention has been directed in this review exclusively to those non-volatile nitrosamines that occur exclusively in tobacco. These nitrosamines are derived from the tertiary amine nicotine and the secondary amines, nornicotine, anatabine and anabasine, which occur exclusively in tobacco and so they called tobacco-specific nitrosamines (TSNAs).

The amounts of TSNAs present varies widely with the nitrite content and the type of tobacco (Fischer *et al.*, 1989) and are typically in the order of a few milligrams per kilogram (Hoffman and Adams, 1981).

A number of studies revealed that two of these nitrosamines—*N*-nitrosonornicotine (NNN) and 4-(methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone

Table 6. Survival and tumour incidence in male F344 rats treated by topical application to the oral mucosa of snuff extracts, or NNN and NNK (from Hecht *et al.*, 1986)

Group	Nitrosamines (μg/application) NNN	NNK	Survival (weeks)	Cheek	Hard palate	Tongue	Lung
Water control	—	—	103 ± 34	—	—	—	(1)
Snuff extract	6.6	1.4	108 ± 24	—	—	—	—
Snuff extract and NNN and NNK	74	15	106 ± 32	(1)	(1)	(1)	(2)
NNN and NNK	60	14	106 ± 39	(6)	(1)	(2)	4 + (1)

Numbers in parentheses indicate benign tumours.

(NNK) are carcinogenic by the oral or parenteral routes in rats and mice. In rats, NNN produces tumours in the nasal cavity and oesophagus while NNK produces tumours in the lung, liver and nasal cavity. In mice, both NNN and NNK produce tumours in lung and liver.

Hecht *et al.* (1986) devised a protocol in which groups of 30 male rats were treated by application to the oral cavity of either aqueous extracts of snuff, aqueous extracts of snuff to which 10 times the naturally occurring concentrations of NNN and NNK had been added, or aqueous solutions of NNN and NNK equal to the amount added to snuff extract (Table 6). Each of these solutions (0.5 ml) was absorbed onto a cotton swab and painted over the oral cavity and lips of rats until the entire amount in the swab had been used. This procedure was carried out once a day for the first 7 days. From week 2 to 23 it was carried out once daily on Tuesdays and Thursdays and twice a day on the other 3 days of the week, and from week 24 to 131 it was twice daily. The experiment was terminated at 131weeks.

No tumours were observed in the group of 30 rats treated with snuff extract. The aqueous solution of NNN and NNK produced a statistically significant increase in benign tumours of the oral mucosa. Snuff extract, with the 10-fold dose of NNN and NNK produced a lower incidence of these benign tumours (three *v.* nine, see Table 6), suggesting, according to the authors, that snuff or some component of it may have an inhibitory effect on the carcinogenicity of these nitrosamines.

A study on the topical carcinogenicity of native snuff was carried out on monkeys (Smith *et al.*, 1970). The oral mucosa was exposed to snuff by various devices for up to 3 to 7 years. No neoplasms were produced in this study by any of these treatments.

Viruses and smokeless tobacco

There are at least two families of viruses that have been implicated in mammalian neoplasia—the herpes viruses and the human papilloma viruses.

Human papilloma viruses (HPV) are a group of small DNA viruses that induce benign skin lesions, including squamous warts and papilloma in man (Münger *et al.*, 1992). Some HPVs have been associated with oral leukoplakia and keratoses of the type found in users of ST (Greer *et al.*, 1987). According to Watts *et al.* (1991), they have been found in approximately 60% of carcinomas from the oral region of the mouth (floor of mouth, tongue, pharynx and larynx). It would thus appear that HPVs might be implicated in the development of oral carcinoma in man. However, there do not appear to be any relevant results from experimental animals which throw light on the extent to which HPVs might be involved in a neoplastic process or on whether external agents can influence their possible activity in this respect.

HSVs form a large group which infect most mammalian species. There are four types that have been implicated in human disease: Epstein Barr Virus (EBV), cytomegaly virus (CMV), varicella-zoster (VZV) and herpes simplex virus (HSV) (Shillitoe and Silverman, 1979). HSV can cause acute and recurrent infections in oral or genital epithelia in humans. HSV is divided into two principal types, HSV-1 and HSV-2 according to the production of small (HSV-1) or large (HSV-2) vesicles when injected into the chorioallantoic membrane of the chick embryo. HSV-1 is generally isolated from the oral cavity epithelium and HSV-2 is primarily found in the genital regions, but the division is not absolute since either virus may be isolated from these anatomical sites (Shillitoe and Silverman, 1979). HSV-1 virus is ubiquitous, and approximately 70% of the adult population in the US and western Europe are seropositive for this virus (Larsson *et al.*, 1989).

In humans, the course of the infection by HSV-1 is often divided into three stages. During the primary stage, vesicular lesions appear and are localized to the mucous membranes but the virus travels back along the sensory nerves towards the regional sensory ganglion, where it may remain latent for several years. Subsequent herpetic infections are principally due to the release of the virus from latency and its passage along the sensory nerves to the skin (Shillitoe and Silverman, 1979).

In preliminary investigations in experimental animals designed to simulate the course of human infection by HSV-1, it was found that a severe inflammatory reaction occurred on first intradermal injection of the virus in the upper lip and it was possible to recover the virus from practically all infected rats during the acute phase. When the reaction subsided, the virus was no longer at the site of inoculation but was dormant in the trigeminal ganglion (Park *et al.*, 1985).

Hirsch *et al.* (1984) investigated the possible role of snuff applied regularly to the mucosa of a surgically-constructed lip canal in rats infected with HSV-1 in producing a neoplastic response. A group of 10 rats was left untreated after the surgical construction of lip canal. A second group of 10 rats had snuff inserted into the lip canal daily for 18 months. A third group was inoculated with HSV-1 virus by monthly scarification of the lip canal without any additional treatment. A fourth group was inoculated with HSV-1 virus and had snuff inserted into the lip canal, with scarification repeated after every month. The experiment was terminated at 18 months. No tumours developed in the group of rats infected with HSV-1 or in the group that had snuff applied to the uninfected lip canal, but in the group infected with HSV-1 and treated with the application of snuff, two carci-

nomas developed. In this investigation, virus was cultured regularly from the oral cavity of inoculated rats while no virus was isolated from the uninfected animals.

The rat lip canal was used by Larsson *et al.* (1989) to compare the actions of snuff as a promoter to virally or chemically "initiated" cells. The part of this report that describes the chemical initiation appears in an earlier part of the review (see p. 1021). In the viral initiation section the authors reported that 55 SD rats were divided into four groups and all had surgery to create an artificial lip canal. Two groups (I and III—see Table 3) were inoculated with HSV-1 once monthly for the duration of the study. Group III then had 200 mg Swedish snuff inserted in the lip canal twice daily 5 days a week for the duration of the study allowing an average exposure of 12 hours per day. Two further groups were included. One group (VI) was left untreated after the construction of the lip canal and the other (group II) received snuff in the same way as group III but had no other treatment. The study was terminated at 30 months. No deaths occurred, but a few rats were killed during the experiment for humane reasons.

Squamous cell carcinomas of the lip canal or close to its orifice were found in one rat in each of groups I and II. There were no tumours of the oral cavity in group III. The occurrence of tumours at other sites appeared to be unrelated to treatment. There was no indication of an interaction between snuff and the virus in this experiment.

In another study designed to investigate the possible interaction between HSV-1 and snuff, Park *et al.* (1986) divided 125 male golden Syrian hamsters into seven groups of 15–20 animals per group as follows:

- Group I received no treatment and served as untreated controls;
- Group II had a mock inoculation (scarificed and swabbed with sterile culture medium);
- Group III had a mock inoculation plus simulated snuff dipping;
- Group IV received HSV-1;
- Group V received HSV- 2 inoculation;
- Group VI received HSV-1 inoculation plus snuff dipping;
- Group VII received HSV-2 inoculation plus snuff dipping.

The viral inoculations were repeated once every 4 weeks for 24 weeks. This elicited a marked reactive lesion which was maintained by the infection with the virus. Approximately 150 mg snuff was placed in each pouch twice a day for 6 months, after which the experiment was terminated. Ten of the 20 hamsters in group VI and and 11 of the 20 in group VII developed invasive squamous cell carcinoma of the cheek pouch. No tumours developed in any of the other groups.

In a subsequent experiment, Park *et al.* (1993) investigated the interaction between HSV-1 and NNN, NNK, or BaP in Syrian hamsters. Groups of 20 Syrian hamsters were inoculated repeatedly (presumably every 4 weeks) with 100 μl culture medium or with the same amount of culture medium containing HSV-1 into the right buccal pouch. The left pouch remained untreated. The site of inoculation was then treated with topical application of 1% NNN, NNK, or with BaP, each dissolved in mineral oil or with mineral oil alone. The topical applications were carried out three times a week for 15 weeks in the case of BaP, and for 20 weeks for NNN, NNK or mineral oil. The experiment was terminated 30 weeks after the first inoculation. There was a low incidence of pouch tumours in the group treated with culture medium and BaP and a higher incidence in the group treated with HSV-1 plus BaP (Table 7).

Comment

Inoculation of HSV-1 in the buccal mucosa of the hamster causes a marked inflammatory reaction characterized by oedema and squamous cell hyperplasia. Because the site has to be reinfected every 4 weeks to maintain continued presence of the virus, the inflammatory reaction is not allowed to subside. The introduction of snuff in an acute in-

Table 7. Effect of tobacco-related chemical carcinogens on HSV1, alone or in combination, on the development of oral cancer in the hamster buccal pouch (from Park *et al.*, 1993)

Group no.	No. of pouches per group	No. of pouches with tumours	Invasive squamous cell carcinoma
1		0*	0
2	20	0	0
3	20	0	0
4	20	0	0
5	20	0	0
6	20	0	0
7	20	4	9
8	18	10	4

Two animals died prematurely of encephalitis in group 8.
Groups 1, 3, 5 and 7 were mock inoculated and then treated topically with mineral oil. (Group 1) or with mineral oil containing 1% of NNK (Gp 3), NNN (Gp 5) or BaP (Gp 7).
Groups 2, 4, 6 and 8 were inoculated with HSV-1 and then treated topically with mineral oil.
(Group 2) or mineral oil containing 1% NNK (Gp 4), NNN (Gp 6) or BaP (Gp 8).
No. of hamsters per group = 20.

flammatory lesion of this sort is bound to cause a marked exacerbation of the oedema and squamous hyperplasia, a type of reaction that is known to predispose to tumour development in rodents.

Summary and Conclusion

No carcinogenic activity was observed when snuff was inserted into the cheek pouch of the hamster or spread over the oral mucosa. This negative result was obtained in a number of experiments whether snuff was applied once only and left in place for several months or inserted repeatedly for up to 2 years.

In the rat, a few tumours were observed when snuff was inserted into the artificial lip canal. The insertion appeared to cause a considerable reaction in the surrounding tissue so it is plausible to assume that trauma plays an important role in the development of these tumours.

An extract of snuff applied to the oral mucosa of the rat did not produce any tumours, but an extract enriched by the addition of 10 times the naturally-occurring amounts of NNN and NNK produced a few benign tumours at the site of application. A higher incidence of tumours was produced when an equivalent amount of an aqueous solution of these two nitrosamines was applied directly to the oral mucosa, suggesting, according to the authors, that snuff inhibits the carcinogenic activity of TSNAs.

Initiation/promotion studies were carried out on snuff in the rat in order to explore further its carcinogenic potential. The results were consistent with the conclusion that snuff does not possess any promotional activity.

No increase in tumour incidence was observed in mice when snuff was given in the diet at concentrations of 25% gradually decreasing to 5% in a 14 month study. A negative result was also obtained in the rat given snuff at a concentration of 5% for 18 months. In hamsters given snuff at a concentration of 20% for 2 years, forestomach tumours occurred. A comparable incidence of this type of tumour occurred in animals given 20% cellulose. The result of this study does not provide valid evidence of carcinogenicity.

HSV and snuff applied orally in the hamster produced a high incidence of squamous cell carcinomas. The sustained high level of squamous cell hyperplasia generated by the experimental design could account for the development of these tumours.

Despite the defects in some of the earlier studies, the sum total of this experimental work suggests that snuff is not carcinogenic to the oral mucosa of the hamster or the rat. It is also unlikely to cause tumours in other tissues in these species. These results give some degree of reassurance that snuff is not likely to be carcinogenic to the human oral mucosa. The interaction of snuff and HSV viruses is, at the moment, questionable and requires further investigation.

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