Journal of Oral Pathology 1984: **13**: 52–62

Effect of snuff and herpes simplex virus-1 on rat oral mucosa: possible associations with the development of squamous cell carcinoma

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The purpose of this investigation was to study the effect of snuff and experimentally induced herpes simplex virus type 1 (HSV-1) infection in Sprague-Dawley rats. It was demonstrated that it was possible to obtain 100% development of acute HSV-1 infection in the rat oral mucosa, but only 10% of latent reactive infection of the trigeminal ganglia. The rats were, therefore, acutely infected monthly with virus to simulate recurrence of latent infection. Virus was applied topically to the mucous membrane twice with an interval of one month. Snuff was administered between the virus applications and afterwards to half the virus-exposed animals. Sham-infected rats were given snuff during the same period (18 months). A fourth group of rats were left untreated. A complete post-mortem examination was performed. Two rats exposed to snuff and HSV-1 in combination developed squamous cell carcinoma of the oral cavity. It was also found that rats exposed to snuff alone or in combination with HSV-1 had a higher incidence of tumours or tumour-like conditions than control rats exposed to HSV-1 only. The incidence of malignant tumours was significantly higher in rats exposed to snuff or HSV-1 and snuff in combination than in control animals ($p < 0.05$). The results of the study indicate that HSV-1 in combination with snuff exposure may be associated with the development of squamous cell carcinomas of the oral cavity.

Accepted for publication May 24, 1983

Snuff-dipping has become an increasingly popular alternative to cigarette smoking, particularly in the southern United States and Scandinavia. Snuff induces in most cases typical lesions of the oral mucosa at the site where the quid is placed (Hirsch et al. 1982). Tobacco habits such as snuff-dipping have been associated with oral leukoplakias (Mehta et al. 1981), and carcinoma (Axéll et al. 1978, Winn et al. 1981). However, the severity of mucosal abnormalities has not been shown to be related to the amount of snuff used or to the time of exposure (Hirsch et al. 1982). This indicates either that there is a great genetic variation in sensitivity to snuff, or that non-inherited factors may contribute to the severity of snuff-induced mucosal abberations.

Herpes simplex virus (HSV) type 1 (HSV-1) may be considered as a possible co-factor for snuff-induced lesions in the mouth. It is an ubiquitous infectious agent

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that not only acutely infects the oral mucosa but persists life-long in a latent state in trigeminal ganglia. In many individuals who harbour HSV-1, the latent trigeminal infection is reactivated at longer or shorter intervals and the virus may subsequently be shed in the oral cavity (Stevens 1975). Recurrent infections with HSV-1 have also been suggested to be aetiologically associated with intra-oral leukoplakias (Lehner et al. 1973), and squamous cell carcinomas (Kvasnicka 1956, Wyburn-Mason 1957, Marshall 1973, Holfinshead et al. 1973, 1976, Shillitoe et al. 1982).

Infection of permissive cells, e.g., oral epithelial cells, with HSV-1 normally results in replication of the virus and death of the cells. If the virus is rendered non-infectious, e.g., by ultra-violet (u.v.) irradiation, it may transform cells *in vitro* (Rapp & Shillitoe 1978). It is, therefore, of interest that extracts of snuff inhibit the replication of HSV-1 *in vitro* (Hirsch et al. 1983, unpublished).

The purpose of this study was to compare the pathological changes of rat oral mucosa after exposure to infectious HSV-1 in combination with long-term administration of snuff with those in animals given HSV-1 or snuff alone. Furthermore, to evaluate the possible carcinogenic effect of infectious HSV-1, snuff and the combination of HSV-1 and snuff.

Material and methods

Green monkey kidney (GMK AH-1) cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% calf serum, 100 i.u. of penicillin and 100 μg of streptomycin per ml. For maintenance, the same medium supplemented with 2% calf serum only was used.

Herpes simplex type 1 (HSV-1) strain F (supplied by Dr Roizman, Chicago USA) was used. Virus was propagated in GMK cells which were infected at a low multiplicity of infection. Cell-associated virus was obtained by lysing cells in a Dounce homogeniser and pooled with overlaying medium. Virus suspensions were spun at low speed and further clarified by centrifuging at 15,000 × g for 10 min. Aliquots of stock virus were kept at −70°C. Virus infectivity was assayed by plaquing on GMK cells grown in 5 cm plastic petri dishes and expressed as plaque-forming units per ml (p.f.u./ml).

Female Sprague-Dawley rats, 3 months old, (Anticimex AB, Stockholm, Sweden) were used. Three to 5 rats were kept in Makrolon cages (No. 3 Jacoby, Stockholm, Sweden) and fed a standard pellet diet (Astra-Ewos AB, Södertälje, Sweden) and water *ad libitum*. The virus-infected rats were kept well separated from the others in a special room in the animal quarters. The temperature was kept between 21 and 23°C and the relative humidity was 40%. The light followed daylight rhythm and was never less than 8 h in length.

The rats were anaesthetised with sodium pentobarbitone (60 mg/ml, 35 mg/kg body weight, intraperitoneal injection), and gently taped with their backs against a table. The virus suspension (0.05 ml) was applied topically to the mucous membrane on the inside of the lower lip after scarification with a 26 gauge needle.

Ether-anaesthetised rats were perfused with Hanks' balanced salt solution through the left cardiac chamber and the brain removed to expose the trigeminal ganglia. The ganglia of the left and right side were removed separately and placed in tubes with cell culture medium at room temperature. The ganglia were minced with scissors and added to cell culture bottle seeded with GMK cells (5×10^5 cells per bottle when the pieces of ganglia were added). Each ganglion was co-cultivated separately. Culture medium was replaced twice weekly. Microscopic readings of cultures

were performed between Days 1 and 28. Virus from cultures demonstrating cytopathic effects (cpe) of GMK cells was passaged on new GMK cultures and typed according to the procedure described by Jeansson (1972). Using this technique (Kristensson et al. 1979) it was possible to reactivate latent HSV from mouse trigeminal ganglia in 75% or more of infected animals as long as 180 days post infection.

Acute HSV-1 infection of rat oral mucosa

Ten rats were inoculated with HSV-1 (2.5×10^7 p.f.u./ml). Another 10 rats were sham-infected. The rats were examined once daily during the following week. Samples for virus isolation were taken on Days 3 and 4. Vesicles presented were perforated with a sterile needle or the mucous membrane was sampled. A sterile sampling rod was pressed and rotated carefully so that cell material was absorbed by the cotton wool. The rod was placed in a tube with cell culture (GMK AH-1) and then removed. The tubes were incubated at 37°C and examined from the third to the seventh day for cpe.

Latency establishment of HSV-1 in rat trigeminal ganglia

Twenty animals were inoculated with virus (HSV-1 2.5×10^7 p.f.u./ml) and 10 rats sham-infected. Three weeks later, the same area of the mucous membrane of the lower lip was scratched in 10 of the infected rats and 10 animals were exposed to UV-light at the site of the infection to provoke recurrence of HSV in the oral mucosa. Ultraviolet irradiation was performed for 10 min using an Osram HNS

Table 1.
Experimental design.

Day	Scarification 0	Virus 0	Saline 0	Snuff 10	Scarification 30	Virus 30	Saline 30	Snuff 40
Control rats (n=10)	*	-	-	-	*	-	-	-
Snuff--exposed rats (n=10)	*	-	*	*	*	-	*	*
HSV--exposed rats (n=10)	*	*	-	-	*	*	-	-
HSV-Snuff--exposed rats (n=10)	*	*	-	*	*	*	-	*

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ri- a- n)	Virus	Saline	Snuff
	30	30	40
	-	-	-
	-	*	*
	*	-	-
	*	-	*

30-W emitter at a distance of 20 cm. Five of the sham-inoculated rats were scratched in the same way and 5 exposed to UV-light.

In a second experiment, the procedure was repeated with the same number of animals (20 + 10) but all rats were given cyclophosphamide (250 mg/kg body weight) 3 days before inoculation of virus. In both experiments the animals were observed daily and samples for virus isolation were taken during the week after recurrence provocation.

In a third experiment, the procedure was repeated using 40 animals. Groups of 10 rats were inoculated using the following titres of the virus: 2.5×10^7, 2.5×10^6, 2.5×10^5 p.f.u./ml and an additional 10 control rats were sham-infected. The rats were killed 30 days after infection and the trigeminal ganglia were removed and co-cultivated.

Long-term effect of snuff on HSV-1-infected rat mucosa

Forty-five rats were operated on under intraperitoneal anaesthesia (sodium pentobarbitone 60 mgl/ml, 35 mg/kg body weight) to create a test canal in the lower lip in which snuff could be retained as described previously (Hirsch & Thilander 1981). Five of these rats were not included in the study, due to suture insufficiency. The remaining 40 animals were then divided into 3 test groups and one control group (Table 1). After scarification with a sterile 26-gauge needle in the test canals, virus (2.5×10^7 p.f.u./ml) was applied topically to the mucous membrane in 20 rats. Ten of the remaining animals were sham-infected with sterile saline solution and 10 left untreated. Snuff was injected into the test canals of 10 virus-infected and the 10 sham-infected rats after 10 days. The snuff (Röda Lacket, Svenska Tobaks AB, Göteborg, Sweden) was applied morning and night, 5 days per week, as described in detail by Hirsch & Thilander (1981). The scarification and application of virus and saline solution was repeated after 1 month in the same animals, after which snuff was injected as before into the test canals after 10 days (Table 1). The rats were given snuff for 18 months, when their condition deteriorated and all of them were killed by injecting sodium pentobarbitone 35 mg/kg body weight, followed by intracardiac aspiration of blood until cardiac arrest occurred.

All rats in the long-term study underwent a complete post-mortem examination. Histological examination was performed on th lip, crevicular epithelium of the lower incisors, heart, kidneys, adrenal glands, urinary bladder, spleen, stomach, small intestine, large intestine, liver, thyroid gland, lungs, brain and

Fig. 1. Acute HSV-1 infection of the rat oral mucosa. A rat with vesicles in the mucous membrane on Day 4, from which positive virus isolation was obtained.

grossly abnormal organs or tissue. The material was fixed in 4% buffered formalin solution and embedded in paraffin. Five micron sections were stained with hematoxylin-eosin and according to the Weigert-van Gieson method.

In order to test for differences in incidence of malignant tumours between the 4 groups of animals, Fisher's exact probability test was used (significance level 5%).

Results

Acute HSV-1 infection of rat oral mucosa

All samples for virus isolation from 10 acutely infected rats, were positive on Day 4. Vesicles were present in 3 of these rats (Fig. 1). Samples obtained from 10 sham-inoculated animals were all negative.

Reactivation of HSV-1 latent infection in rat trigeminal ganglia

The results of the 2 experiments aiming at obtaining recurrent HSV-1 infection in the rat oral mucosa by scratching or by UV irradiation were negative. No samples for virus isolation obtained during one week showed presence of virus. In the third experiment, where the animals were inoculated with different titres of HSV-1, reactivation of virus by co-cultivation of trigeminal ganglia was obtained in one of the rats in the group exposed to the most concentrated virus suspension.

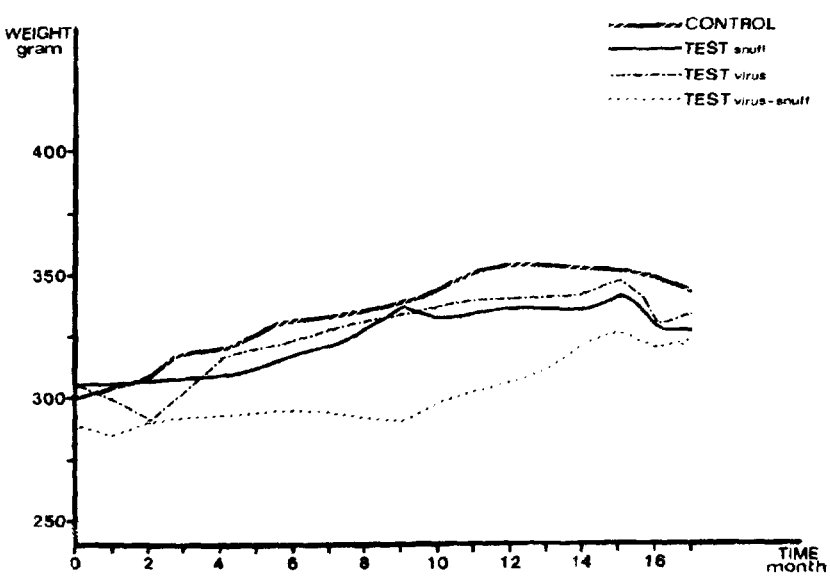

Fig. 2. Body-weight curves for test and control animals during the experiment. After the second application of virus, 3 rats in the virus-exposed and an equal number in the virus-snuff-exposed group showed weight loss.

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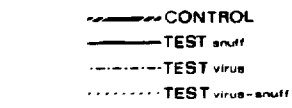

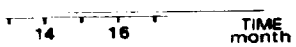

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virus-snuff-exposed group showed weight

Table 2.
The incidence, degree and distribution of histopathological lesions in the lip mucosa of control, snuff, virus and snuff-virus-exposed Sprague-Dawley rats. I = slight; II = moderate; III = severe.

		1. Controls (n=10)	2. Snuff exposure (n=10)	3. HSV exposure (n=7)	4. HSV-snuff exposure (n=7)
Atrophy	I		8	3	1
	II				
	III				
Ulceration	I		1	1	3
	II				
	III				
Hyperortho-keratosis	I	5	9	7	4
	II				2
	III				1
Vacuolation	I	5	4	2	2
	II				
	III				
Hyperplasia	I	5	5	6	2
	II		2		3
	III				1
Acanthosis	I		2		
	II		2	1	5
	III				1
Dysplasia	I		3		4
	II				
	III				
Inflamma-tion	I	3	4	2	3
	II				1
	III		1		1
Fibrosis	I	4			
	II	2			
	III		10	7	7

Long-term effect of snuff on HSV-1-infected mucosa

The above results showed that it was possible to obtain 100% development of acute HSV-1 mucosal infection without any mortality but at the most, 10% of latent reactive infection of

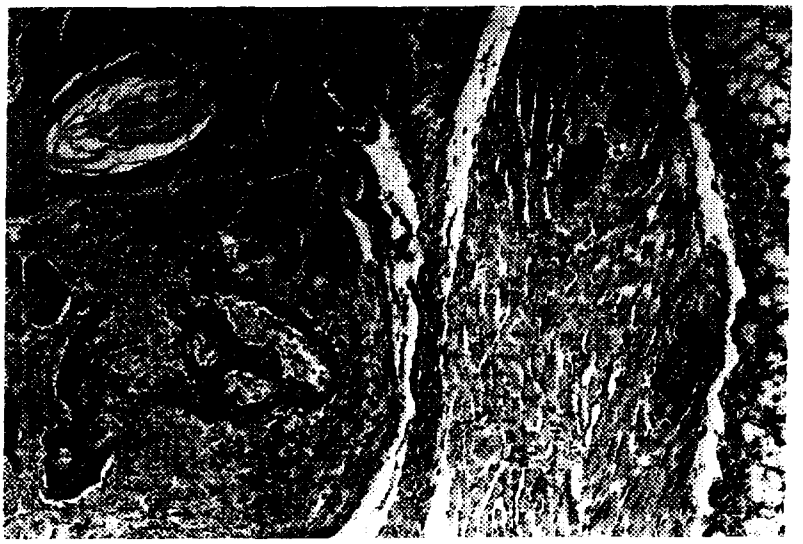

Fig. 3. Moderately well-differentiated squamous cell carcinoma of the oral cavity, invading bone and striated muscle. (H&E ×92).

the trigeminal ganglia. Therefore, we decided that rats should be acutely infected with concentrated virus (2.5×10^7 p.f.u./ml) monthly to simulate recurrence of latent infection.

After the second application of virus, 3 rats in each of the virus- and snuff-exposed groups were in poor condition, showed loss of weight (Fig. 2), decreased activity, and finally died from encephalitis. Application of virus was, therefore, not further repeated. The other rats remained in good health for 18 months from the start of the experiment, after which their condition deteriorated and all rats were killed.

Morphological findings in the oral cavity and mucosa

The incidence, degree and distribution of histopathological lesions in the lip mucosa of control, snuff, virus, and snuff-virus-exposed rats are shown in Table 2.

Control group. The squamous epithelium of the control rats was mildly hyperplastic (50%) and covered with an orthokeratin layer (50%). The inflammatory reaction was slight or absent and the fibrosis slight to moderate.

Virus group. The squamous epithelium was usually slightly hyperplastic with areas of atrophy. The surface layers consisted of hyperorthokeratinised cells. Dysplasia was not found and inflammatory reactions in the connective tissue were absent or very mild. Severe fibrosis was noted in all specimens.

Snuff group. In this group the epithelium was, in general, somewhat more hyperplastic (mild to moderate), hyperorthokeratinised and showed areas of slight atrophy. Vacuolated cells were seen, as well as acanthosis and mild inflammation. Mild dysplasia was noted in 30% of the rats and all animals exhibited severe fibrosis.

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Virus-snuff group. The epithelium in this group was, generally, moderately hyperplastic and covered with a hyperorthokeratotic cell layer. Ulcerated lesions were found in almost 50% of the specimens, as well as slight to moderate acanthosis. The inflammatory reaction was more pronounced in this group, and more than 50% of the specimens exhibited mild dysplasia of the squamous epithelium. Severe fibrosis was noted in all cases. Macroscopic tumours were found in 2 rats ($p < 0.07$). The tumours were detected when the rats were killed at the nd of the test period. They were ulcerated and one tumour

Table 3.
The type, incidence and distribution of tumours or tumour-like lesions among the test and control rats.

Type and site of histopathological lesion	1. Controls (n=10)	2. Snuff exposure (n=10)	3. HSV exposure (n=7)	4. HSV-snuff exposure (n=7)
Follicular Tumour				
Thyroid gland	8	8	7	5
Squamous cell carcinoma				
Oral cavity				2
Anal region		1		
Sarcoma				
Retroperitoneum		1		1
Papillary Squamous hyperplasia				
Forestomach		5		2
Fibroadenoma				
Breast	3	3	1	3
Cystic Cholangioma				
Liver			1	1
Adenofibroma				
Ovary				1
Phaeochromocytoma				
Adrenal gland			1	
Desmoplastic fibroma				
Skin				1
Total number of tumours	11/10	18/10	10/7	16/7

situated on the palatal side of the right molar region of the upper jaw. The other tumour was found on the lingual side in the molar region, also on the right side. Both tumours were moderately well-differentiated squamous cell carcinomas invading the bone (Fig. 3). Mild to moderate dysplasia was noted in the crevicular epithelium of the lower incisors in the virus (40%), snuff (10%), and virus-snuff group (86%).

Morphological lesions outside the oral cavity

In 10 rats exposed to snuff only, 18 tumours or tumour-like conditions were detected. The corresponding incidence in the control rats was 11 tumours in 10 rats. The 7 HSV-infected rats had 10 tumours, and 7 rats exposed to HSV and snuff had 16 tumours or tumour-like conditions. Papillary squamous hyperplasia of the forestomach was detected in 5 of 10 rats exposed to snuff only, and in 2 of 7 exposed to both HSV and snuff (Table 3). Furthermore, one rat exposed to snuff alone developed a moderately well-differentiated squamous cell carcinoma of the anus. One rat in the same group, and one in the combined HSV and snuff group, developed a poorly differentiated retroperitoneal sarcoma. Three rats in Group 2 and an equal number in Group 4 had mild to moderate epithelial hyperplasia of the urinary bladder. Follicular tumours of the thyroid gland were frequent in both test and control rats. Snuff-exposed rats (snuff alone and snuff-HSV) had a significantly higher incidence of malignant tumours than control rats and rats exposed to HSV alone ($p < 0.05$).

Discussion

The histopathological findings in the oral cavity of the HSV-1-exposed rats were insignificant. In comparison with those in rats in which test canals had been created in the lips and then left withot further treatment. The rats whose test canals had merely been exposed to snuff exhibited lesions which were comparable with those described in earlier experiments with snuff expoure (Hirsch & Thilander 1981, Hirsch & Johansson 1983). The third test group, which had been exposed to both HSV-1 and snuff, exhibited the most pronounced histopathological changes. Ulcerated and dysplastic lesions were, thus, more frequent, but most notable was that 2 of 7 rats had developed squamous cell carcinomas of the oral cavity. These 2 macroscopic tumours were found in close contact with the crevicular epithelium. Even if the tumours were not detected at the exact site of the application of the 2 agents, it is reasonable to assume that they were caused by exposure to HSV-1 and snuff, or snuff derivatives in the saliva. The crevicular epithelium has earlier been reported (Lekholm 1976) to have a weak protective capacity against chemical substances. In a previous study comprising 52 snuff-exposed rats, one rat developed a similar tumour after 8.5 months (Hirsch & Johansson 1983).

Experimental data in support of the carcinogenic potential of snuff are not conclusive. Bioassays *in vivo* for carcinogenicity from different types of snuff implantations have been found to be negative (Hirsch & Thilander 1981). In the present study, the rats exposed to snuff alone or in combination with HSV, had a higher incidence of tumours or tumour-like conditions than the control rats or rats exposed to HSV only. The control rats only exhibited follicular tumours of the thyroid gland or fibroadenomas, which were also found among the test groups. Malignant tumours only developed among the rats exposed to snuff, alone or in combination with HSV.

In a previous communication, we have reported on the presence of papillary squamous

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on the presence of papillary squamous hyperplasia of the forestomach after snuff exposure (Hirsch & Johansson 1983). This lesion was also found in the present study among rats exposed to snuff alone or in combination with HSV. Even though the incidences are low, they may indicate that snuff has tumorogenic properties, possibly related to its content of tobacco-specific nitrosamines (Hoffmann & Adams 1981). Although there are no animal experiments which have demonstrated a direct carcinogenic effect of infectious HSV *in vivo*, a co-carcinogenic potential has been shown (Southam et al. 1969, Burns & Murray 1981). Duff & Rapp (1971) found that if the cytolytic properties of HSV type 2 (HSV-2) were inhibited, the virus was capable of transforming hamster cells *in vitro*. When transplanted back into the original host, these cells grew as carcinomas. The capacity of both the HSV-1 and HSV-2 genomes to induce cell transformation *in vitro* has since been shown in a number of studies (Rapp & Turner 1978). Burns and Murray (1981) recently reported on the development of squamous cell carcinomas in mouse lips after HSV-2 infection in combination with u.v.-exposure and tetradecanoyl-phorbol-acetate (TPA) application. They suggested that the *in vivo* u.v.-irradiated HSV-2 acted as the inducer and TPA as the promoter in a classical two-stage carcinogenesis model.

The results of this study indicate that HSV-1 in combination with snuff exposure may also be associated with the development of squamous cell carcinomas of the oral cavity. It is possible that the snuff acts as a co-carcinogenic substance due to its restrictive effects on cytolytic HSV-1 infections, a prerequisite for virus-induced malignant transformation (Hirsch et al. 1983). The capacity of snuff extracts to inhibit cytolytic HSV-1 infection might be related to their content of nitrosamines as such substances have been reported to inhibit HSV replication *in vitro* (Roane 1978). This would incriminate the nitrosamines in tobacco in 2 ways: as direct inducers of malignancy or as indirect inhibitors of HSV infection, and thereby, facilitating transformation of cells by HSV. This study is now being repeated and extended and attempts will be made to find HSV-1 antigens and/or detect virus genes in the malignant cells.

Acknowledgements

The study was in part supported by the Swedish Cancer Society, project No. 1657-B83-03X. The authors thank Miss Lena Brändén and Miss Annika Dahlqvist for excellent technical and secretarial help.

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