Oral cancer induced in hamsters with herpes simplex infection and simulated snuff dipping

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A number of epidemiologic studies indicate that snuff dipping is associated with an increased incidence of oral cancer in human beings. Since inactivated herpes simplex virus (HSV) has been shown to induce malignant changes in vitro and in vivo and is partially inactivated by snuff water extract, we examined the histopathologic changes of hamster buccal pouches after exposure to repeated HSV inoculation combined with long-term simulated snuff dipping. One hundred twenty-five Syrian hamsters were divided into seven groups, and the buccal pouches were inoculated with HSV-1, HSV-2, or culture medium. The mock and HSV inoculations were done once a month for 6 consecutive months. In an effort to determine the effect of snuff on the mock- or HSV-inoculated buccal pouches, a consistent amount of a commercially available snuff was placed into both the right and left pouches twice a day in half of the animals. At the end of the 6 months of simulated snuff dipping (4 weeks after the final mock or viral inoculation), the hamsters were killed and the buccal pouches were removed for the histopathologic evaluation. Neither simulated snuff dipping nor HSV infection alone induced neoplastic changes in hamster buccal pouches. However, HSV infection in combination with simulated snuff dipping resulted in epithelial dysplasia and invasive squamous cell carcinoma in more than 50% of the animals.

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A number of epidemiologic studies indicate that the use of snuff (snuff dipping) is associated with an increased incidence of oral cancer in human beings.[1-4] However, experimental data are lacking on the effects of snuff dipping on oral mucosa, and no animal studies have demonstrated the neoplastic changes in animal oral cavity with simulated snuff dipping.[5] Therefore, possible involvement of other contributing factors must be considered in the development of malignant oral lesions related to the use of smokeless tobacco.

Herpes simplex virus (HSV) has been shown to have a link to certain human malignant tumors.[6-10] Patients with oral cancer tend to have high titers of neutralizing antibody to type 1 HSV (HSV-1).[10] Many epidemiologic and molecular biologic studies

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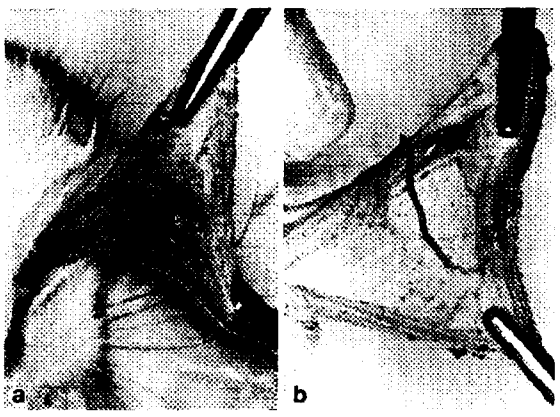

Fig. 1. HSV-1 inoculated hamster buccal pouches. **a**, Three days after the viral inoculation only, presenting extensive erythema, edema, and vesicle formation. **b**, Three days after the viral inoculation with simulated snuff-dipping, showing absence of virus-associated clinical lesions.

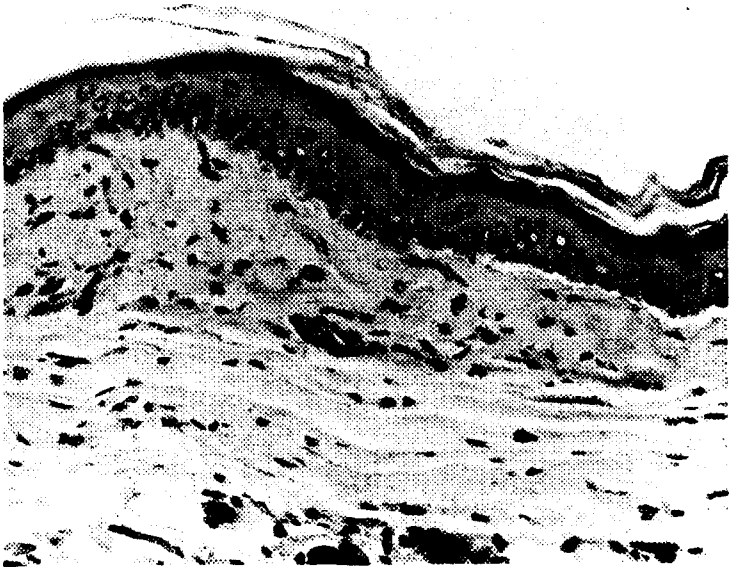

Fig. 2. Photomicrograph shows normal hamster buccal pouch mucosa exposed to snuff for 6 months. The stratified squamous epithelium is of normal thickness, with regular orientation of underlying fibrous connective tissue. (Hematoxylin and eosin stain. Original magnification, ×200.)

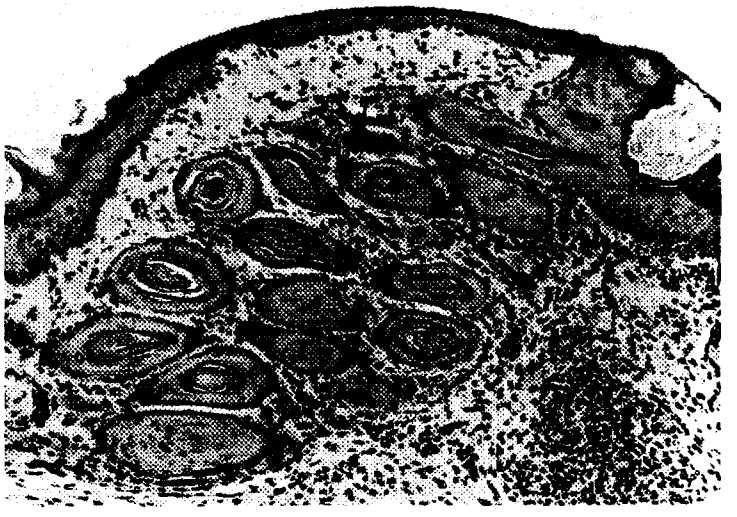

Fig. 3. Photomicrograph shows hamster buccal pouch mucosa infected with HSV-2 and exposed to snuff for 6 months. The epithelium reveals hyperkeratosis, acanthosis, and severe dysplasia as evidenced by epithelial proliferation into the underlying connective tissue. Epithelial islands exhibit keratin pearl formation, pleomorphism, reversal of nuclear-cytoplasmic ratio, loss of polarity, and prominent nucleoli. Chronic inflammatory infiltrate consisting predominantly of lymphocytes is seen interspersed around the epithelial islands. (Hematoxylin and eosin stain. Original magnification, ×100.)

have suggested that there is a distinct link between human uterine cervical cancer and type 2 HSV (HSV-2).[6-9] Experimentally, cellular transformation has been demonstrated with ultraviolet-irradiated HSV, fragments of viral DNA, and photodynamically inactivated virus in vitro.[11-14] HSV inactivated by ultraviolet light or formalin was demonstrated to induce uterine cancer in mice.[15] However, to show cellular transformation or cancer induction, prior inactivation of the otherwise rapidly cell-lysing HSV was required.

Recently water-extracted constituents of snuff

Table I. Effect of HSV infection and simulated snuff dipping on the histopathologic changes in hamster buccal pouches

Experimental groups	*Histopathologic changes**				
	Inflammatory infiltrate in lamina propria	*Hyperkeratosis*	*Hyperplasia*	*Epithelial dysplasia*	*Invasive squamous cell carcinoma*
1. No treatment	0/15	8/15	0/15	0/15	0/15
2. Mock inoculation	0/15	9/15	0/15	0/15	0/15
3. Mock inoculation plus simulated snuff dipping	2/15	15/15†	8/15†	0/15	0/15
4. HSV-1 inoculation	3/19	19/19†	8/19†	0/19	0/19
5. HSV-2 inoculation	11/16[b]	16/16†	13/16†	3/16	0/16
6. HSV-1 inoculation plus simulated snuff dipping	14/20[b]	20/20†	20/20†	12/20†,‡	10/20†,‡
7. HSV-2 inoculation plus simulated snuff dipping	15/20[b]	20/20†	20/20†	13/20†,‡	11/20†,‡

*Numerator: Number of animals with the described histopathologic changes. Denominator: Number of animals examined.
†Significantly different ($P < 0.05$) from no treatment or mock inoculation groups (Fisher's exact test, double tail).
‡Significantly increased ($P < 0.05$) compared to HSV inoculation-only group (Fisher's exact test, double tail).

were reported to inhibit the replication of HSV in vitro.[16] More recently, we reported that a water extract of snuff was capable of inactivating HSV in cell-free conditions and inhibiting the replication of HSV inoculated in the tissues of hamster buccal pouches.[17] In the present study we examined the histopathologic changes of hamster buccal mucosa after exposure to intermittent HSV inoculations combined with long-term simulated snuff dipping.

MATERIALS AND METHODS

Animals

One hundred twenty-five male golden Syrian hamsters *(Mesocricetus auratus)* were purchased from the Simonson Laboratories, Gilroy, California. The animals were randomly bred, 6 weeks old, and weighed approximately 100 grams.

Virus

HSV-1, F-strain (from the American Type Culture Collection [ATCC], Rockville, Maryland) and HSV-2, G-strain (from ATCC) were used throughout the experiment. They were grown in primary rabbit kidney (PRK) cells, with viral titers determined by using the plaque assay technique.[18] The virus stock was stored at −80° C until used in the present study.

Inoculation of hamster buccal pouches with HSV

With the animals under general anesthesia with ketamine HCl (Parke-Davis Warner-Lambert Co., Morris Plains, N.J.), 100 mg/kg of body weight, intramuscular injection, both right and left buccal pouches were scratched with a 26-gauge needle in a cross-hatched pattern over a 1.0 cm^2 area. Then 50 μl of HSV-1 (F-strain, 5×10^5 PFU per pouch) or HSV-2 (G-strain, 5×10^3 PFU per pouch) was applied topically over the scratched area with gentle massage. The viral infection was repeated once every 4 weeks for six cycles. Mock inoculation (control) was done with culture medium instead of virus-containing solution.

Intraoral administration of snuff (simulated snuff dipping

A commercially available snuff was purchased. A consistent amount of snuff (approximately 150 mg per pouch) was placed into the right and left buccal pouches twice a day by means of a hollow plastic cylinder with a central rod permitting the tobacco to be pushed to the end of the cylinder with a single stroke.

Experimental groups

The hamsters were divided into seven groups of fifteen to twenty animals each, as follows: (1) no treatment, (2) mock inoculation, (3) mock inoculation plus simulated snuff dipping, (4) HSV-1 inoculation only, (5) HSV-2 inoculation only, (6) HSV-1 inoculation plus simulated snuff dipping, and (7) HSV-2 inoculation plus simulated snuff dipping.

In groups 3, 6, and 7, simulated snuff-dipping was initiated 24 hours after the first HSV inoculation. The snuff was placed twice a day, 5 days per week, for 6 consecutive months. The hamster pouches were observed twice a week for clinical changes.

Clinical and histopathologic findings of pouch mucosa

The buccal pouches of animals were observed throughout the experiment to record the development of keratosis, erythema, tumor nodules, and the

size and number of tumor masses. At the end of the experimental period (4 weeks after the last mock or HSV infection), the animals were killed and the buccal pouches were removed. The pouches were grossly examined and fixed in a 10% neutral formalin, sectioned in paraffin, and stained with hematoxylin and eosin for light microscopic examination. Unless there was invasive cancer, the histologic changes were graded according to a modified version of a histologic classification of oral leukoplakias as proposed by Kramer and colleagues.[19] An assessment was made of the following features: keratinization (normal, increased); granular cell layer thickening (absent, present); acanthosis (absent, present); epithelial dysplasia (absent, present). Epithelial dysplasia was recorded if the epithelial cells exhibited loss of polarity, bizarre mitoses, nuclear hyperchromatism, or cellular pleomorphism.

RESULTS

Clinical changes of hamster buccal pouches

The developmental pattern of intraoral HSV lesions in hamster buccal pouches was similar to that reported earlier.[20] When the hamster buccal pouches were inoculated with HSV-1 or HSV-2 only, the inoculated area became erythematous on the first postinoculation day, and edema appeared on the second postinoculation day. Small vesicles and ulcer formation were evident on the third postinoculation day. Ulceration, erythema, swelling, and vesicles reached their peak on the third or fourth postinoculation day and began to decline sharply thereafter. After postinoculation day 8 there were no evident virus-associated clinical lesions. However, one animal from Group 4 (HSV-1 only) and four animals from Group 5 (HSV-2 only) died of HSV encephalitis between 14 and 20 days after the first viral inoculation. In the animals with simulated snuff dipping with HSV inoculation, the development of clinical lesions was very mild compared to that of the HSV-inoculation only group. In fact, vesicles and ulceration did not develop when snuff was placed in the pouches (Fig. 1). At the end of the experimental period, all buccal pouches of animals in Groups 1, 2, 3, 4, and 5 appeared normal. The animals appeared healthy, and no gross pathologic changes were discovered at autopsy. The animals in Groups 6 and 7 also appeared healthy and no pathologic changes were observed at autopsy, but the thickness of HSV-infected portions of the buccal pouch tissues was somewhat increased in comparison to those of the animals in Groups 1 to 5.

Histopathologic findings

Table I shows the histopathologic effect of snuff, HSV inoculation, and combined snuff and HSV inoculation. In the control groups (no treatment or mock inoculation only), no animals showed inflammatory infiltration, epithelial hyperplasia, epithelial dysplasia, or invasive cancer in buccal pouches. Fifty-three percent of the no treatment group and 60% of the mock infection group of animals exhibited hyperkeratosis. Simulated snuff dipping in mock-inoculated animals significantly increased the number of animals with hyperkeratosis and hyperplasia, but none exhibited epithelial dysplasia or cancer. An inflammatory infiltrate was seen in 16% and 69% of the animals inoculated with HSV-1 and HSV-2, respectively. Furthermore, HSV inoculation significantly increased the number of animals with hyperkeratosis and hyperplasia in comparison to the controls. Although HSV-2 inoculation induced epithelial dysplasia in 19% of the animals, it did not induce invasive cancer in the pouches. HSV-1 or HSV-2 infection in combination with simulated snuff dipping resulted in rather distinct histopathologic changes of the oral mucosa: More than 60% of animals showed epithelial dysplasia and 50% to 55% of the animals (25% to 30% of the pouches) revealed invasive squamous cell carcinoma. Figs. 2 and 3 show photomicrographs representative of mucosa with snuff alone and buccal mucosa altered by HSV-2 infection in combination with simulated snuff dipping, respectively.

DISCUSSION

Although snuff is thought to have carcinogenic properties, our data suggest that snuff alone does not induce precancerous or neoplastic changes in hamster buccal pouches. Although snuff cannot be shown to be a direct carcinogen when kept in apposition to oral mucosa for 6-month periods, when combined with a repeated HSV infection it resulted in the development of precancerous or/and cancerous changes in more than 50% of the tested animals. Therefore, it appears that snuff may act as a cocarcinogenic substance by its inhibitory action on cytolysis due to HSV infection. When HSV is inactivated and has lost its cytolytic activity by certain chemicals or ultraviolet irradiation, it can cause neoplastic changes in cells and animal tissues.[12,15] Clinically, the buccal pouches with infection combined with snuff showed mild reactive changes, while those with HSV infection alone developed severe inflammatory lesions. Moreover, none of the animals that received snuff died, in spite of the repeated HSV infection. These data strongly suggest that simulated snuff dipping into buccal pouches resulted in the inhibition of the cytolytic action of HSV in buccal pouch tissues. However, since repeated HSV infection alone also induced precancerous changes and simulated snuff dipping alone did not cause the dysplastic

changes in the pouch tissues, HSV may be more responsible for the development of neoplastic changes. The reactivation of HSV in the trigeminal ganglia and a concomitant shedding of the virus into the tissues of the human oral cavity in a patient with recurrent herpes labialis or herpetic stomatitis are not infrequent events.[21, 22] Therefore, snuff dipping might pose a potential danger for the development of malignant oral conditions in human beings with frequent severe recurrent intraoral HSV lesions.

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