

Inhibition of Herpes Simplex Virus Replication by Tobacco Extracts¹

Jan-Michaél Hirsch, Bo Svennerholm, and Anders Vahne²

Departments of Oral Surgery, Faculty of Odontology [J.-M. H.], and of Virology, Institute of Medical Microbiology [B. S., A. V.], University of Göteborg, Göteborg, Sweden

ABSTRACT

Herpes simplex virus type 1 (HSV-1) has been associated with the genesis of leukoplakias, epithelial atypia, and oral cancer. Tobacco habits, such as snuff dipping, are also definitely correlated with this type of lesion. The normal cytolytic HSV-1 infection can, after *in vitro* inactivation, transform cells. Extracts of snuff were prepared and assayed for their ability to inhibit HSV-1 replication. Plaque formation assays of HSV-1 in the presence of snuff extract showed that a reduced number of plaques was formed. Different batches of one brand of snuff were tested for inhibition of herpes simplex virus (HSV) production. More than 99% inhibition of 24-hr HSV production was obtained with undiluted batches. The 1:5 dilutions of snuff had an inhibitory effect of 85% and 1:25 dilutions, 39%. In agreement, the attachment of the virus to the host cell and penetration of the virus to the cell nuclei were found to be inhibited as was the synthesis of viral DNA. Nicotine had an inhibitory effect, while aromatic additions to snuff were found to have no major inhibitory effect on HSV replication. Snuff extracts were prepared from different brands of snuff reported to contain high and low quantities of tobacco-specific *N*-nitrosamines. Brands with reported high levels of tobacco-specific *N*-nitrosamines had significantly greater ability to inhibit HSV replication. In conclusion, this study has shown that extracts of snuff have inhibitory effects on the production of cytolytic HSV-1 infections. A chronic snuff dipper keeps tobacco in the mouth for the major part of the day. Thus, virus shed in the oral cavity in connection with a reactivated latent HSV-1 infection has great possibilities of being affected by snuff or derivatives of snuff. It is suggested that an interaction between tobacco products and HSV-1 might be involved in the development of dysplastic lesions in the oral cavity.

INTRODUCTION

Circumstantial evidence links HSV³ with cancer in humans (for review, see Ref. 25). Most scientific efforts have been concentrated on the possible association of HSV-2 with the development of uterine cervical carcinoma. However, HSV has also been associated with the genesis of intraoral leukoplakias and epithelial cell atypia (12), squamous cell carcinoma of the oral cavity (12, 25), and cancer within the head and neck (27, 28).

The oncogenic capacity of HSV is well underlined. Both HSV-

1 and HSV-2 can transform cells *in vitro*. When such cells are transplanted to the homologous host (hamster, mouse, or rat), they may form malignant tumors that frequently metastasize (for review, see Refs. 15 and 19). However, a prerequisite for HSV to cause cell transformation is that the virus-induced cell lysis is prevented. UV irradiation of the virus, photodynamic inactivation with neutral red, change of environmental temperature, or the use of HSV temperature-sensitive mutants are methods that have been used to inhibit HSV cell lysis in transformation studies *in vitro* (for review, see Ref. 20). Recently, Burns and Murray (3) reported that herpetic lesions of mouse lips could be converted to malignant squamous cell carcinomas by the use of tetradecanoylphorbol acetate as tumor-promoting agent, if the virus was inactivated previously by UV irradiation *in vivo*.

Reactivation of HSV in the trigeminal ganglia and peripheral shedding of virus in the mouth are, in many people, frequent events not necessarily accompanied by epithelial lesions (4). Therefore, substances which inhibit HSV replication and which are held in the mouth for prolonged periods of time might be of potential danger for the development of oral cancers.

Besides the association with HSV-1, an increased risk for acquisition of intraoral leukoplakias (14) and oral cancer (36) has been attributed to the popular use of tobacco in the mouth.

We have therefore considered the possibility of a joint action in oncogenesis (29) of HSV and substances found in tobacco. Recently, we reported on the development of squamous cell carcinoma in the oral cavity of rats after HSV-1 infection of the oral mucosa in combination with prolonged exposure to snuff (7). A correlation between the use of tobacco (smoking), HSV, and oral cancer on the basis of HSV-neutralizing antibody levels has also been reported by Shillitoe *et al.* (24).

In this paper, we have studied the effects of water-extractable compounds of snuff on HSV replication *in vitro*.

MATERIALS AND METHODS

Cells. Monkey kidney cells (GMK AH-1 and Vero cells) were cultured as monolayers with Eagle's MEM supplemented with 10% calf serum, 100 µg of streptomycin/ml, and 100 IU of penicillin/ml (growth medium). The same medium supplemented with only 2% serum was used as maintenance medium. Cell counting was performed as described previously (33).

Viruses. HSV-1 strain F (supplied by Dr. B. Roizman, Chicago, IL), the Edmonston strain of measles virus (NIH research reference strain), and coxsackie B5 virus (ET 652) were used. Techniques for preparation of virus stocks and for plaquing of virus in GMK cells (HSV-1 and coxsackie B5 virus) or in Vero cells (measles virus) have been described in detail elsewhere (13, 35). Preparations of purified HSV labeled in the DNA with [³H]thymidine were performed as described previously (30).

Snuff Extracts. Water extracts of snuff were prepared from 3 different brands of snuff (Brand 1, Röda Locket; Brand 2, Ettan; and Brand 3, Tre Ankare). The snuff was kindly supplied fresh by Svenska Tobaks AB, Göteborg, Sweden, and stored in a refrigerator (-40°) prior to use. In addition to tobacco "leaves," Brand 1 contains salt, water, and flavoring

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² To whom requests for reprints should be addressed, at Department of Virology, Institute of Medical Microbiology, University of Göteborg, Guldhedsgatan 10 B, S-413 46 Göteborg, Sweden.

³ The abbreviations used are: HSV, herpes simplex virus; HSV-2, herpes simplex virus type 2; HSV-1, herpes simplex virus type 1; TSNA, tobacco-specific *N*-nitrosamines; GMK, green monkey kidney; Hanks' BSS, Hanks' balanced salt solution; TCA, trichloroacetic acid; MEM, minimal essential medium; MOI, multiplicity of infection; PFU, plaque-forming units; CPE, cytopathic effects.

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substances. Information about the exact nature of these substances is not obtainable from the manufacturer. Brand 2 is devoid of such additions. These 2 brands are delivered in waxed cardboard containers with plastic lids. The third brand, with snuff portions individually wrapped in aluminum foil, is a Swedish brand introduced recently, known to be free of volatile *N*-nitrosamine and to contain relatively low levels of TSNA (2). The first 2 brands contain relatively high quantities of volatile *N*-nitrosamine and TSNA (2). To study the possible effect of ageing of snuff (Brand 1), the lid was removed, and the container was exposed to open air for 14 days. Such a procedure has demonstrated earlier an increase of TSNA in snuff (8).

To prepare the extract, 10 g of snuff were mixed with 50 ml of Eagle's MEM, incubated at 37° for 1 hr, and centrifuged twice at 1000 rpm for 10 min. The resulting supernatant was used after pH adjustment to 7.2 by 0.1 M HCl, whereupon the solution was made sterile by filtration through a Millipore filter (0.22 µm). The nicotine concentration of 2 batches of snuff extracts (Brands 1 and 2) was determined according to a method described earlier (10). The Brand 1 extract contained 0.8 mg/ml, and the Brand 2 extracts contained 1.2 mg/ml (67 and 75% of the total content of nicotine extracted, respectively). Pure nicotine was kindly supplied by Svenska Tobaks AB, Stockholm, Sweden.

Cell Growth Assays. Bottles with monolayers of GMK cells were trypsinized, and the cells were suspended in growth medium and counted. Aliquots of 4.0 ml of the cell suspension (5×10^4 cells) were placed in 5-cm plastic Petri dishes ($n = 48$), and 0.5 ml of a test solution (Eagle's MEM or Brand 1 snuff extract at a dilution of 1:1, 1:5, or 1:25 in Eagle's MEM) was added to each culture. Cell counts were then performed after 24 and 48 hr of incubation at 37° (6 cultures with each test solution were harvested at each time point).

Cellular DNA replication was assayed by growing the GMK cells in the presence of [³H]thymidine. To each of 48 cultures prepared as above, 20 µCi of [³H]thymidine (TRK 120; Amersham, England) were added. After incubation at 37° for 24, 48, and 72 hr, 4 cultures with respective test solutions were washed 3 times with Hanks' BSS and frozen at -70°. The cells were thawed and suspended in ice-cold TCA, 10% (w/v). Precipitable material was collected by centrifugation at $1200 \times g$ for 10 min. The pellets obtained were washed once with TCA, and radioactivity was assayed by liquid scintillation.

Assays of Virus Production. GMK cell monolayer cultures in 5-cm plastic Petri dishes were inoculated with 0.5 ml of virus suspension (MOI of 1 PFU/cell). Virus was allowed to adsorb for 1 hr at 37°. The cells were then washed 4 times with prewarmed Hanks' BSS, and a total volume of 2 ml of the test substances in maintenance medium was added. Control cultures received 2 ml of maintenance medium only. After incubation at 37° for 24 hr, the dishes were frozen at -70°. The dishes were thawed, and the cells were scraped into the overlying medium and tested for virus production by plaque titrations. The above procedure was used for determination of measles virus progeny, with the exceptions that the dishes were incubated for 48 hr and that Vero cells were used.

Assay of HSV DNA Replication. In these experiments, we took advantage of the fact that labeled thymidine is incorporated mainly into HSV DNA if the nucleoside is added to infected cells 6 hr after infection or later (11, 23, 32).

Confluent GMK cell monolayer cultures in 5-cm Petri dishes were infected with 0.5 ml of HSV-1 (MOI, 1 PFU/cell). After adsorption for 1 hr at 37°, the cells were washed 4 times with prewarmed Hanks' BSS, and 3 ml of Brand 1 snuff extract were diluted 1:10 in Eagle's MEM, or 3 ml of Eagle's MEM (controls) were added. The dishes were reincubated at 37° for 5 hr, whereafter 20 µCi of [³H]thymidine were added to each culture. Immediately and 6, 14, and 22 hr after the radioactivity was added, 4 test and 4 control dishes were frozen at -70°. Total TCA-precipitable radioactivity was assayed as described above for radiolabeled cellular DNA.

Adsorption of HSV. An enzyme-linked immunosorbent assay-based technique was utilized for studies on HSV attachment to GMK cells.⁴

Briefly, confluent monolayer cultures were allowed to adsorb HSV at a multiplicity of 100 PFU/cell. After intervals ranging from 0 to 120 min, virus suspensions were discarded, and cells were washed 5 times with 0.1 M phosphate-buffered saline (pH 7.2). The amount of cell-associated virus was assayed by means of sequential adsorption of antibodies against HSV, horseradish peroxidase-conjugated anti-IgG, and final addition of substrate. Adsorption curves were plotted on the basis of absorbance at 492 nm versus the time of attachment.

Assay of Radiolabeled Virus in Fractions Containing Cell Nuclei. The processing of virus to the nuclei of infected cells was determined as described previously (34). Monolayer cultures of GMK cells were inoculated with 2.0×10^6 cpm of the virus preparation. After adsorption at 37° for 1 hr, the cells were washed 5 times in Hanks' BSS. The cells were then incubated at 37° with desired medium (control, Brand 1 snuff extract at 1:1 and 1:10 dilutions) for 5 hr to allow transfer of attached virus to nuclei. Thereafter, the cells were scraped off with a rubber policeman, and nuclei and cytoplasmic fractions were prepared by the method of Penman (17). The percentage of cell-associated radioactivity found in the nuclei was calculated.

Assay of Interferon. A sensitive bioimmunoassay developed recently (5) was used, determining reduction in the amount of vesicular stomatitis virus antigens produced in human lung carcinoma cells (A549 cells; American Type Culture Collection, CCL185) or GMK cells in presence of material to be tested. Cultures of human embryonic lung cells and GMK cells were exposed to snuff extract (Brand 1 extract diluted 1:5 in maintenance medium) for 10 hr at 37°. The cells were then washed 3 times with Hanks' BSS and reincubated with maintenance medium for 12 hr. The cultures were thereafter freeze-thawed, and cell debris was removed by low-speed centrifugation. The remaining supernatants were tested for interferon content. A human interferon of known activity was included in each test. The sensitivity of the test was < 1 IU/ml.

RESULTS

Effect of Snuff Extracts on Cell Growth. Confluent cultures of GMK cells in Petri dishes were incubated at 37° with snuff extract in various concentrations (Brand 1, 1:1, 1:2, 1:4, 1:8, 1:16, and 1:32 in Eagle's MEM, and as control Eagle's MEM) in duplicates. The cells were read in a light microscope over 6 subsequent days for toxic effects. Only at the 2 highest concentrations of the snuff extract could morphological signs of snuff-induced toxicity be seen, first appearing on Days 5 and 6. To study effects of snuff extract on the growth rate of GMK cells, cells suspended in growth medium were seeded to plastic Petri dishes, and 1:10 volume of snuff extracts (Brand 1 extract diluted 1:1, 1:5, and 1:25 in Eagle's MEM and as control Eagle's MEM) was added. The cultures were incubated at 37°, and cell counts were performed after 24 and 48 hr of incubation. A slight reduction of cell growth was obtained in cultures receiving the highest concentration of snuff extract, but no inhibitory influence was noted with the more diluted test solution. The 1:25 dilution rather seemed to stimulate cell growth (Chart 1A). Cell toxicity was also tested by studying cellular DNA replication in the presence of snuff extracts by means of [³H]thymidine incorporation (Chart 1B). As was observed in the cell-counting assay, only the 1:1 dilution of the snuff extract had a slight inhibitory effect. Again, the 1:25 dilution of the snuff extract exhibited a potentiating effect as measured by the incorporation of radioactivity.

Effects of Snuff Extracts on HSV Infectivity. HSV suspensions (2×10^7 PFU/ml) were mixed with equal volumes of different dilutions of a Brand 1 extract. Immediately and after 60 min of incubation at 37° or at room temperature, duplicate samples were drawn and assayed for residual infectivity. Virus

⁴ B. Svennerholm, manuscript in preparation.

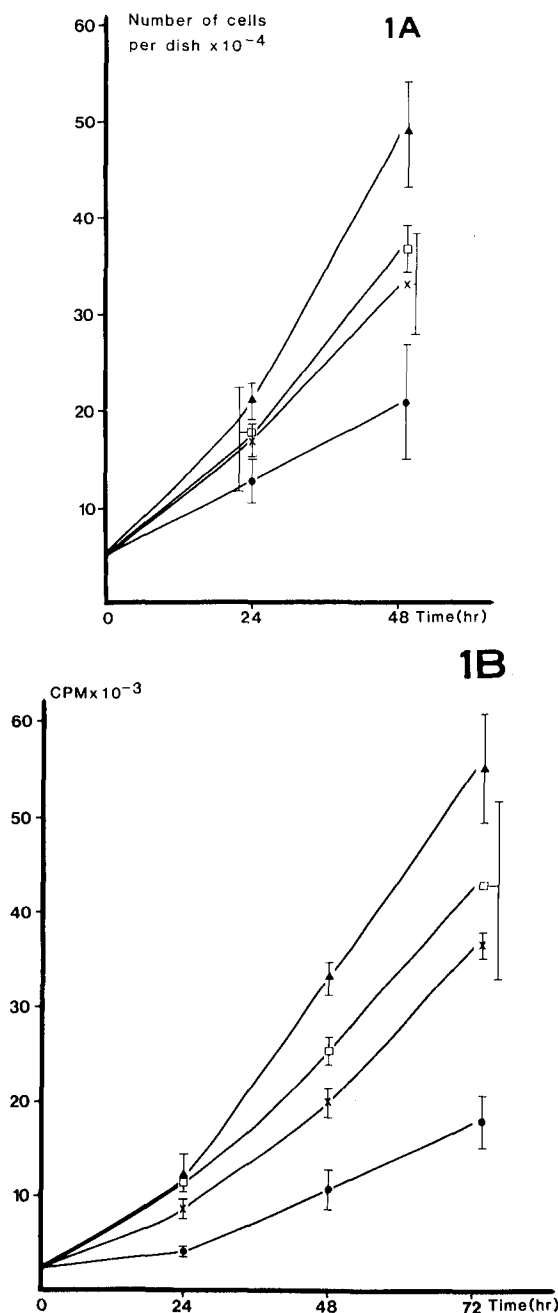


Chart 1. The effect of snuff extract on GMK cell growth. One-half ml of Brand 1 snuff extract diluted 1:1, 1:5, or 1:25 in Eagle's MEM and Eagle's MEM as control were added to 5-cm Petri dishes ($n = 96$), with 4 ml of cell suspension each (5×10^4 cells). In A, cell counts were performed after 24 and 48 hr of incubation at 37° ($n = 48$). Points, mean of 6 cultures; bars, S.E. ●, 1:1; ×, 1:5; ▲, 1:25; □, control. In B, to assay the effect on cellular DNA replication, $20 \mu\text{Ci}$ of [^3H]thymidine were added to the tissue cultures ($n = 48$) prior to incubation at 37° for 24, 48, and 72 hr. The cells were harvested, pelleted, and assayed by liquid scintillation for radioactivity. Points, mean of 4 cultures; bars, S.E. ●, 1:1; ×, 1:5; ▲, 1:25; □, control.

suspensions mixed with Eagle's MEM served as controls. No loss of virus infectivity other than the temperature inactivation seen in the controls was obtained with snuff extracts diluted 1:5 or more at either temperature. However, undiluted snuff extract induced a slight (20%) inactivation of HSV when incubated at

Effect of Snuff Extract on HSV Attachment to Cells. The kinetics of the attachment of HSV to cellular receptors in presence of varying concentrations of snuff extracts was studied. Mixtures of HSV and extracts of snuff (Brand 1) at a 1:1 or 1:50 dilution were added to monolayers of GMK cells. After intervals ranging from 0 to 120 min, the cells were washed, and the amount of cell-associated virus was determined (Chart 2). A complete inhibition of the attachment of HSV in the presence of the highest concentration of snuff (1:1) was encountered. Snuff extracts diluted 1:50 resulted in a significantly lower uptake rate of HSV as compared to the snuff-free virus suspensions.

To examine the influence of snuff extracts on later stages of the replicative cycle of HSV, snuff was excluded during the attachment period in the following series of experiments.

HSV Replication in the Presence of Snuff Extracts. The influence of snuff extracts on HSV in cell culture was first tested by studying virus plaque formation in the presence of the extracts. Monolayers of GMK cells in 5-cm Petri dishes were infected with 10-fold serial dilutions of HSV. After 1 hr of adsorption, the virus inoculum was replaced with maintenance medium containing 1% methylcellulose and a 1:10, 1:50, or no concentration of a Brand 1 extract. The number of plaques was read on Day 5 of incubation at 37° . The 1:10 dilution reduced the number of plaques to 9%, and the 1:50 dilution, to 43% of those found in the control cultures.

The inhibitory effect of the snuff extract on HSV replication was also tested by assaying 24-hr virus yields in cells incubated in the presence or absence of the extract after infection. In 5 experiments, 5 different batches of Brand 1 extracts were tested. In each experiment, GMK cell cultures (5-cm Petri dishes) were infected with HSV at MOI of 1 PFU/cell. After 1 hr of adsorption, the cells were washed 4 times with prewarmed Hanks' BSS and then incubated with dilutions of snuff extracts in maintenance medium. Control cultures received maintenance medium only. More than 99% inhibition of 24-hr HSV production was obtained with all 5 undiluted batches of snuff extracts (Table 1). At dilutions

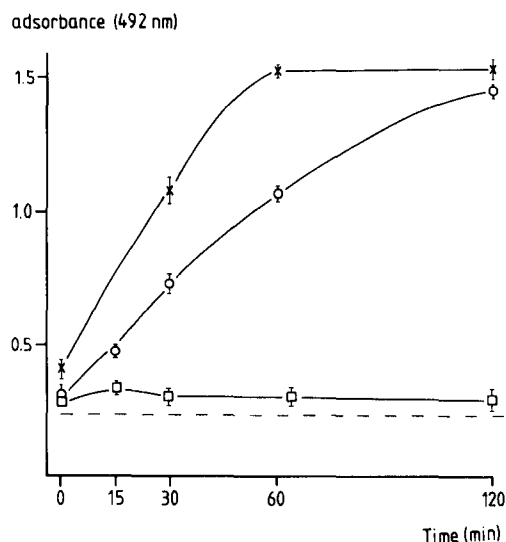


Chart 2. Adsorption of HSV to GMK cells in presence of snuff diluted 1:1 (□) and 1:50 (O) or in the absence of snuff (×). The amount of cell-associated virus, indicated by absorbance at 492 nm, is plotted versus the time of attachment.

of 1:5, the inhibitory effect of the different batches varied between 65 and 98%, and 1:25 dilutions gave inhibitions even less (15 to 72%) as compared to yields of the controls.

The effect of removal of snuff extract from drug-treated HSV-infected cells was tested. GMK cell cultures were infected at MOIs of 0.05, 0.5, and 5 PFU/cell. After 1 hr of adsorption at 37°, the cells were washed as above and reincubated with a 1:5 dilution of snuff extract (Brand 1) in maintenance medium. Twenty-four hr postinfection, the cultures were washed 3 times in prewarmed Hanks' BSS and reincubated with maintenance medium alone. The cell cultures were read daily for the appearance of HSV-characteristic CPE. Infected cultures that were not exposed to snuff extracts but otherwise treated similarly served as controls. In these latter cultures, CPE were detected 24 hr

Table 1
Inhibition of HSV production by snuff extract

Batch	% of control at following dilution of snuff extract		
	1:1	1:5	1:25
1	<1 ^a	3 ^a	28 ^a
2	<1	4	48
3	<1	13	56
4	<1	35	86
5	<1	20	85
	0.2 ± 0.2 ^b	15 ± 6	61 ± 11

^a Mean of 3 cultures.

^b Mean ± S.E.

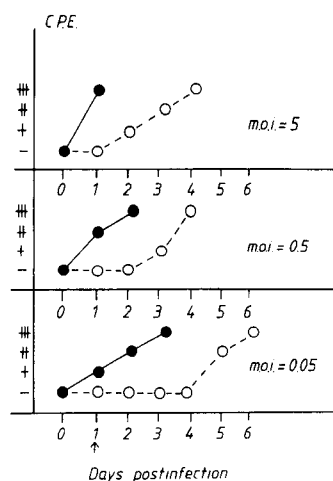


Chart 3. Appearance of HSV CPE in infected GMK cell cultures after removal of snuff extract. GMK cells were infected with HSV at an MOI of 0.05, 0.5, or 5 PFU/cell. After adsorption for 1 hr, the cells were washed and reincubated for 23 hr in the presence of a 1:5 dilution of a snuff extract (○). After 24 hr (arrow), the cultures were released from the snuff extract and read daily for HSV-specific CPE. Sham-treated cultures (●) served as controls. The CPE were scored as follows: —, no CPE detected; +, up to 30%; ++, 30 to 60%; and +++, more than 60% of the cells showing CPE. Each point represents the mean score of 3 cultures.

postinfection (Chart 3). None of the snuff extract-treated cultures showed signs of CPE at the time of removal of the drug. At various times thereafter, depending on the MOI used, scattered cells in the cultures showed morphological signs of HSV infection. In the cultures infected at 0.05 PFU/cell, HSV CPE were not detected before 4 days after release from snuff exposure. Virus-induced CPE then gradually spread to finally comprise all cells of the cultures (Chart 3).

A Brand 1 extract (Batch 3) was also tested for effects on yields of coxsackie B5 virus and measles virus. As seen in Table 2, the production of both of these viruses was reduced to about the same extent as HSV by the snuff extract. Thus, although these extracts seemed to have little or no cell-toxic effects, their inhibitory effect on virus production was not specific for HSV or DNA viruses. However, no interferon activity was detected in cell cultures treated with the snuff extract.

Effect of Snuff Extract on HSV DNA Synthesis. To determine if the block in HSV production was on an early or late function of the virus replication cycle, synthesis of HSV DNA in the presence of snuff extracts was assayed. TCA-precipitable radioactivity was assayed in GMK cell cultures which had received [³H]thymidine 6 hr postinoculation and which were incubated with the labeled nucleoside for various periods of time.

Under these experimental conditions, most radioactivity will be incorporated into viral DNA (11, 23, 32). The results are shown in Chart 4. The Brand 1 extract at a 1:10 dilution significantly reduced the incorporation of [³H]thymidine into the TCA-precipitable material. Thus, the snuff-induced block in HSV replication appears to be on an early function, i.e., before or at the level of DNA replication.

Effect of Snuff Extract on Penetration and Transport of HSV to the Cell Nuclei. As the inhibition of HSV replication appeared to be an early event, the processing of cell-associated radiolabeled HSV to the nuclei of GMK cells in presence of snuff was studied. Monolayer cell cultures were infected with radioactivity labeled and purified virus, which was allowed to adsorb for 1 hr. The cells were then washed and reincubated with Eagle's MEM or extracts of snuff (Brand 1) at a 1:1 or 1:10 dilution. Immediately and after 5 hr of incubation, cells were harvested, and the radioactivity in the nuclei and cytoplasmic fractions was assayed (Table 3). The percentage of total radioactivity found in the nuclear fraction after 5 hr at 37° indicated that incubation with snuff extract reduced the penetration rate of HSV. The least radiolabeled HSV DNA in the nuclear preparations was found in cells incubated with the higher concentrations of the snuff extract.

Effect of Different Snuff Extracts and Nicotine on Virus Production. The main constituents of snuff are tobacco leaves, water, and salt. To achieve various brands, dry flavor ingredients together with an "aromatic juice" are added. Information about

Table 2
Inhibition of production of HSV, coxsackie, and measles virus by snuff extract

Cell virus	Log PFU/ml at following dilution of snuff extract			
	1:1	1:5	1:25	Controls
GMK HSV	2.81 ± 0.03 ^a (99) ^b	5.91 ± 0.58 (87)	6.47 ± 0.56 (44)	6.81 ± 0.25 (0)
GMK coxsackie	4.80 ± 0.01 (98)	7.38 ± 0.16 (37)	7.39 ± 1.2 (34)	7.53 ± 0.21 (0)
Vero measles	1.60 ± 0.31 (99)	2.89 ± 1.5 (76)	4.19 ± 0.46 (78)	5.17 ± 0.07 (0)

^a Mean ± S.E.

^b Numbers in parentheses, percentage of inhibition of virus production.

the exact composition of these latter substances is not obtainable. To determine if there were any main differences in the inhibitory effect on HSV replication of snuff extracts with or without the addition of flavor ingredients and other aromatic substances, snuff extracts were prepared from 2 brands of snuff with (Brand 1) and without (Brand 2) such ingredients. We also tested if the inhibitory effect of snuff could be ascribed to nicotine.

In uninfected GMK cell cultures, no morphological signs of nicotine-induced cell toxicity at the concentrations of 0.6 mg/ml and less were observed during a 4-day observation period. Therefore, a concentration of nicotine of 0.6 mg/ml was tested for HSV-inhibitory effect, together with snuff extract of Brands 1 and 2 in the dilution of 1:10. These brands contained significantly less nicotine (0.08 and 0.12 mg/ml, respectively). The test solutions were added to 5 infected GMK cell culture dishes, respectively. The cultures were incubated at 37° for 24 hr and assayed for production of progeny virus as described earlier.

Nicotine had an inhibitory effect on HSV production, but the inhibitory effect of the snuff extracts cannot be ascribed to this substance alone as demonstrated here (Table 4). It is also shown that the chemicals added to Brand 1 do not significantly influence the inhibitory effect of the snuff extract.

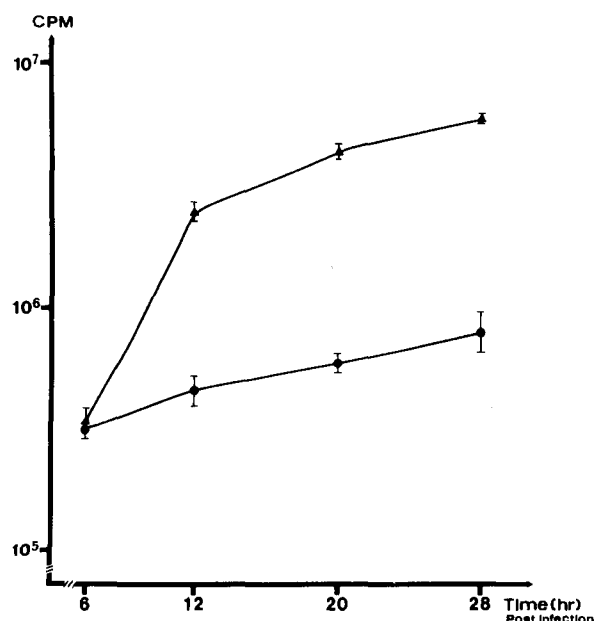


Chart 4. The effect of snuff extract on the production of HSV-1 DNA. Confluent GMK cell monolayer cultures in 5-cm Petri dishes were infected with HSV-1 (MOI of 1 PFU/cell). After adsorption (1 hr) of virus, 3 ml of Brand 1 snuff extract diluted in Eagle's MEM were added (or Eagle's MEM as control). The dishes were reincubated at 37° (5 hr), whereafter 20 μ Ci of [³H]thymidine were added to each culture. Immediately and 6, 14, and 22 hr after addition of the [³H]thymidine, 4 test and 4 control dishes were assayed for radiolabeled DNA. ●, test; ▲, control. Points, mean of 4 cultures; bars, S.E.

Table 3

Inhibition of radiolabeled HSV penetration to cell nuclei by snuff extract

Test substance	Inhibition (%)		
	0 hr	5 hr	Relative increase (0-5 hr)
Control	23.9	58.7	2.5
Snuff (1:10)	26.0	43.3	1.7
Snuff (1:1)	29.9	35.6	1.2

Table 4

Inhibition of production of HSV by nicotine and 2 brands of snuff

Test substance	Log PFU/ml	% of inhibition	Content of nicotine (mg/ml)
Control	7.01 \pm 0.09 ^a	0	
Nicotine	6.67 \pm 0.09	54	0.6
Brand 1	5.61 \pm 0.04	96	0.08
Brand 2	5.79 \pm 0.1	94	0.12
Brand 1 + nicotine	5.07 \pm 0.05	99	0.68

^a Mean \pm S.E.

Table 5

Inhibition of production of HSV by 2 fresh brands of snuff and aged snuff (1:5 dilution)

Test substance	Log PFU/ml	% of inhibition
Experiment 1		
Control	7.95 \pm 0.12 ^a	0
Fresh Brand 1	6.48 \pm 0.16	97
Fresh Brand 3	7.04 \pm 0.06	86
Experiment 2		
Control	7.44 \pm 0.05	0
Fresh Brand 1	6.64 \pm 0.12	84
Aged Brand 1	5.16 \pm 0.008	99.5

^a Mean \pm S.E.

Effect of Fresh and Aged Snuff Extract on Virus Production.

Snuff contains high quantities of TSNA (8). Ageing of snuff in the open air leads to formation of additional TSNA (8). Recently, a new brand of snuff supplied in snuff portions wrapped in aluminum foil was introduced. This snuff (Brand 3) contains significantly less quantities of nitrosamines (2). In order to investigate effects of snuff known to contain high and low quantities of nitrosamines, extracts of Brands 1 and 3 were compared for inhibitory effects on HSV replication. In a second experiment, Brand 1 was allowed to stand exposed to open air for 14 days for additional formation of TSNA before water extraction. From the data presented in Table 5, it is seen that the aluminum-wrapped snuff had less inhibitory effect on HSV replication than did Brand 1. From the second experiment, it can be concluded that ageing of the snuff significantly increased the inhibitory effect of the snuff extract.

DISCUSSION

The tobacco (snuff) water extracts used in this study did not inactivate HSV but were found to inhibit the replication of this DNA virus dose dependently in *in vitro*-cultured GMK cells. One possibility is that these extracts directly or indirectly specifically inhibited a vital virus-coded function. At the concentrations used, the snuff extracts were not toxic to the GMK cells as judged by morphological appearance in the light microscope, cellular growth rate, and thymidine incorporation. However, no specific cellular function was studied. Our snuff extracts are complex mixtures and probably contain many substances with biological activity. For example, many of the now more than 3000 compounds identified in tobacco and tobacco smoke have been shown to increase the permeability of fibroblast plasma membranes (31) and to influence the noradrenaline-induced oxidative metabolism in brown fat cells (18). Therefore, the observed inhibition of HSV in the present study may be due to impairment of cellular functions necessary for HSV replication. Furthermore, the effects observed were not exclusive for HSV. The replication of both an enveloped (measles) and naked (Coxsackie B5) RNA

virus was decreased to the same extent as HSV in the presence of the tobacco extracts. These effects could not, however, be ascribed to interferon induction in snuff extract-treated cells.

The tobacco-induced block in HSV replication was found to be on an early virus function, *i.e.*, before or at the level of virus DNA synthesis. The early events studied, adsorption and processing of attached virus to the cell nucleus, were found to be impaired by snuff. The negative effect of snuff extract on HSV adsorption was not responsible for the restriction of virus replication observed in our experiments, as virus was allowed to adsorb in the absence of the extracts. Whether or not impairment of HSV DNA transport to the nucleus alone was responsible for the observed inhibition of virus cannot be judged from our experiments. Again, due to the complex nature of the extracts, different compounds may influence more than one viral function. The following finding supports this caution. Nontoxic concentrations of nicotine, the one biologically active tobacco compound available to us, restricted HSV replication but not to the same extent as did the snuff extracts, although the concentration tested (0.6 mg/ml) was approximately 5 times that of the extracts. Thus, nicotine is probably one, but not the only, substance contributing to the overall HSV restriction observed with snuff extracts.

To the snuff brand used in most of our studies, as to most other popular brands, flavor ingredients and aromatic substances secret but to the manufacturer had been added. However, these substances probably had little or no influence on HSV replication, as one "pure" brand devoid of such additions restricted HSV equally well as "unpure" brands.

Snuff compounds that recently have attracted much interest are the TSNA. These substances are formed during manufacture of snuff and are major carcinogens (9). Analyses of the content of TSNA in popular Swedish snuff brands have been published recently (8). We compared the 2 brands which were the extremes with regard to TSNA content. Brand 1 contains more than 6 times as much TSNA as does Brand 3 (8). A significantly higher reduction of HSV replication was obtained with the Brand 1 extract. Aging of snuff is a process which increases the amount of TSNA in snuff (8). We tested snuff extracts prepared from fresh and aged (open air for 14 days) snuff from the same snuff batch. Also, this experiment indicated that TSNA might contribute to the antiviral effect of our extracts. Recently, Roane (21) reported on HSV restriction by 2 other nitrosamines (dimethylnitrosamine and diethylnitrosamine).

A chronic snuff dipper keeps tobacco in the mouth for approximately 10 hr daily (mean of 3 investigations; Refs. 1, 6, and 22). Thus, there are constantly great possibilities for virus shed in the oral cavity to be affected by snuff or snuff derivatives. Of the adult population, 80 to 90% have been exposed to HSV-1 (16). Recurrent lesions are experienced by approximately 40% (26), and these occur despite the presence of circulating HSV-neutralizing antibody (4). Thus, interaction between tobacco products and HSV-1 in the development of dysplastic lesions in the oral cavity is a tempting suggestion.

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