Archs oral Biol. Vol. 37, No. 11, pp. 969–978, 1992
Printed in Great Britain.

0003-9969/92 $5.00 + 0.00

INHIBITION OF HERPES SIMPLEX VIRUS REPLICATION AND PROTEIN SYNTHESIS BY NON-SMOKED TOBACCO, TOBACCO ALKALOIDS AND NITROSAMINES

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(Accepted 15 May 1992)

Summary—Inhibitory effects of snuff extract and the tobacco chemicals nicotine, anabasine, diethyl-*N*-nitrosamine (DEN), and the tobacco-specific nitrosamines (TSNA), *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) on herpes simplex virus type 1 (HSV-1) replication *in vitro* and on HSV-1 protein synthesis in infected cells were analysed. Snuff extract and nicotine caused a significant reduction of HSV-1 attachment to cell membranes whereas anabasine, DEN, NNN and NNK did not affect adsorption of HSV-1. Virus production assays in the presence of snuff added after virus adsorption resulted in a significantly reduced production of virus at low multiplicities of infection (MOI), but at high MOI the inhibitory effect of snuff extract was less pronounced. DEN, NNN and NNK only affected virus production at toxic concentrations. Nicotine and anabasine reduced virus production in non-toxic doses but not at the concentrations present in snuff extract. In HSV-infected cells exposed to snuff extract, the immediate early (α-) infected cell proteins (ICPs) 4 and 27 (as well as the early (β-) ICPs 6 and 8) were markedly increased, whereas the late (γ-) ICPs 5, 11 and 29 were reduced. Nicotine had a less pronounced stimulating effect on the production of α-proteins but no detectable effect on production of β- or γ-proteins. Anabasine, DEN, NNN and NNK did not affect HSV protein synthesis at non-toxic concentrations. Synthesis of thymidine kinase and DNA polymerase was significantly reduced by snuff extract. Also nicotine and anabasine affected thymidine kinase and DNA polymerase but only at toxic concentrations. The production of the cellular protein actin, which almost disappears a few hours after HSV-1 infection, remained at a significant level in HSV-infected cells exposed to snuff. Thus snuff extract blocks the replicative cycle of HSV at an early stage, which results in an increased production of α-proteins in the infected cells and in prolonged maintenance of cellular functions. This may be of importance for HSV-induced transformation and the development of HSV-associated tumours.

Key words: herpes simplex virus, protein synthesis, tobacco.

INTRODUCTION

The oncogenic capacity of HSV has been thoroughly investigated. Both HSV-1 and HSV-2 can transform cells *in vitro* (Rapp, 1980; MacNab, 1987; Maitland, 1988). However, a prerequisite for HSV to cause cell transformation is that the virus-induced cell lysis is prevented. The interaction of tobacco extracts with HSV-1 replication has also been studied earlier. Aqueous extract of snuff and condensate, smoked tobacco tar, has been shown to inhibit in a dose-dependent manner, the replication of HSV-1 cultured cells (Hirsch, Svennerholm and Vahlne, 1984b, Stich *et al.*, 1987; Oh, Paik and Park, 1989). Substances that inhibit HSV replication and that are held in the mouth for prolonged periods of time may be of potential danger for the development of malignancies.

Tobacco is associated with an increased risk of developing intraoral leukoplakias (Mehta, Gupta and Pindborg, 1981). Oral cancer has been attributed to the use of tobacco, i.e. both smoking and snuff dipping (Winn, 1984). Although snuff by itself might induce tumours in the oral region, the carcinogenic effect is weak (Hecht *et al.*, 1986; Johansson *et al.*, 1989; Brunnemann *et al.*, 1987). However, the combination of HSV-1 and snuff has a pronounced carcinogenic effect in rats (Larsson *et al.*, 1989) and hamsters (Park, Sapp and Herbosa 1986). The block in HSV replication induced by both a non-smoked tobacco extract (Hirsch *et al.*, 1984b; Oh, Cherrick and Park, 1990) as well as a smoked tobacco tar extract (Oh *et al.*, 1989) is an early function; i.e. before or at the level of DNA replication.

Our aim now was to characterize further the influence of snuff extract and the water-soluble alkaloids, nicotine and anabasine, as well as the carcinogenic tobacco-specific nitrosamines, NNN and NNK, on HSV-1 replication *in vitro* and the production of HSV-infected cell proteins *in vitro*.

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Abbreviations: DEN, diethyl-*N*-nitrosamine; ELISA, enzyme-linked immunosorbent assay; GMK, green monkey kidney; HPLC, high-performance liquid chromatography; HSV, herpes simplex virus; ICP, infected cell proteins; MEM, minimal essential medium; MOI, multiplicity of infection; NNK (see abstract); NNN, *N*-nitrosonornicotine; PBS, phosphate-buffered saline; PFU, plaque forming units; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCA, trichloracetic acid; TSNA, tobacco-specific nitrosamine.

MATERIALS AND METHODS

Cells

GMK and human epidermoid carcinoma (HEp-2) cells were used. The cells were grown as monolayers on 5-cm plastic Petri dishes (NUNC, Denmark) in Eagle's MEM supplemented with 10% fetal calf serum to grow GMK cells and with 8% fetal calf serum to grow HEp-2 cells. Sixty μg of penicillin and 100 μg of streptomycin were added per ml medium. The same media were supplemented with antibiotics, but only 3% fetal calf serum was used for maintenance. The cells were grown in darkness and in an atmosphere of 95% air and 5% CO_2 at 37°C.

Virus

The HSV-1 strains F and MacIntyre were used. The F strain was used for the studies of protein synthesis in infected cells and the MacIntyre strain was used for studies of attachment and viral replication. The techniques for the preparation of virus stock suspensions and for plaquing the virus in GMK cells have been described earlier (Vahlne, Nilheden and Svennerholm, 1981).

Chemicals

Preparation of snuff extract. Water extract was prepared from one brand of fresh Swedish snuff purchased on the open market in Sweden. Ten grams of snuff were mixed with 50 ml of MEM, incubated at 37°C for 1 h, and centrifuged twice at 1000 rev/min for 10 min. The resulting supernatant was used after pH adjustment to 7.4 by 0.1 M HCl and sterilization by filtration through a Millipore filter (pore size 0.22 μm). This aqueous extract of snuff was analysed as to the content of the tobacco alkaloids, nicotine, anabasine and anatabine, and the tobacco-specific nitrosamines NNN and NNK (Johansson *et al.*, 1989). Water extracts were frozen at −40°C before analysis. The results of these analyses are shown in Table 1.

Tobacco chemicals. Nicotine, anabasine and DEN were purchased from Sigma, St Louis, MO, U.S.A. NNN and NNK were kind gifts from Dr D. Hoffmann (American Health Foundation, New York, U.S.A.). NNN and NNK were more than 99.5% pure according to gas chromatography, HPLC, thin-layer chromatography, and mass spectrometry (Hoffmann *et al.*, 1979).

Radiochemicals. L-[^{35}S]-methionine with specific activity of approx. 200 mCi/mmol was purchased from Amersham International, Amersham, U.K. and [^{125}I]-5-iodo-2′deoxyuridine ([^{125}I]-IdUrd) was purchased from New England Nuclear Corp., Cambridge, MA, U.S.A.

Assays of cell toxicity

Cell toxicity of tobacco alkaloids, DEN, NNN and NNK was assayed by three different methods. First, to study the toxic effects on the morphological appearance of GMK cells, confluent cultures of GMK cells were incubated with snuff extract and tobacco chemicals in various concentrations dissolved in maintenance medium. Snuff extract was added at dilutions of 1:5 and 1:25. Nicotine, anabasine, DEN, NNN and NNK were added in the concentrations of 1.0, 0.1 and 0.01 mg/ml. Maintenance medium served as control. The cells were viewed in a light microscope over six subsequent days for toxic effects. Second, to study the effects of snuff extract and tobacco chemicals on the growth rate of GMK cells, 1×10^6 cells were seeded in 5-cm Petri dishes, suspended in growth medium to which snuff extract was added to give the final dilutions of 1:5 and 1:25. Nicotine, anabasine, DEN, NNN and NNK were added yielding the concentrations of 1.0, 0.1, and 0.01 mg/ml. Cells suspended in growth medium served as controls. The cultures were incubated for 72 h, after which the cells were trypsinized and counted on a Coulter counter. Third, the effect of drugs on cell protein synthesis was assessed by the dye-binding assay described earlier by Bradford (1976). Cells suspended in growth medium with drugs added as described above were seeded on Petri dishes. After 3 days the cells were rinsed five times with PBS ($Na_2H_2PO_4$ buffered NaCl 0.155 M, pH 7.4) and scraped off with a rubber plunger. Cells were dissolved in 0.5 ml PBS. This solution was ultrasonicated for 6 min and filtered through a Whatman gf/c filter. Dye-reagent (Bio-Rad, Richmond, CA, U.S.A.) was added, and absorbance was read at 595 nm in a Philips PU 8625 spectrophotometer. Adsorbance values were compared to those obtained from control cultures with growth medium only.

Assay of HSV attachment to cells

GMK cells were grown as confluent monolayers in a 96-well microtitre plate and allowed to adsorb HSV at an MOI of 500 PFU/cell. Snuff extracts (at dilutions 1:1, 1:2, 1:5, 1:25), nicotine, anabasine, NNN, NNK and DEN (at concentrations 1.0, 0.5, 0.1 0.01 and 0.01 mg/ml) were mixed with virus suspensions immediately before inoculation. HSV suspended in Eagle's MEM served as control. After intervals ranging from 0 to 120 min, virus suspensions were discarded, and the cells were washed five times

Table 1. Analytical profile of tobacco alkaloids and tobacco-specific nitrosamines of the Swedish snuff extract used in the experiments

Sample	pH*	Nicotine (μg/ml)	Anabasine (μg/ml)	NNN (μg/ml)	NNK (μg/ml)
1	7.7	913	10.6	1.59	0.51
2	7.7	754	9.6	2.12	0.51
3	7.7	505	4.5	1.47	0.46
4	7.7	585	3.2	1.71	0.52
5	7.6	1090	11.6	1.47	0.48
Mean ± SE		769 ± 106.8	7.9 ± 1.69	1.67 ± 0.121	0.50 ± 0.011

*Prior to pH adjustment.

Table 2. Effect of snuff extract on cellular protein production and cell growth in green monkey kidney cells

Dilution	mg proteins /culture (mean ± SE)	Millions of cells /culture (mean ± SE)
1:5	0.15 ± 0.01*	1.42 ± 0.16
1:25	0.26 ± 0.01	2.39 ± 0.21
Controls	0.29 ± 0.01	2.10 ± 0.16

*Denotes significant difference as compared with controls.

with 0.1 M PBS and fixed in 0.02% formaldehyde. Adsorption of virus was determined by ELISA. After three washes in PBS–Tween 20 (0.05%), 100 μl of the HSV-1 mouse monoclonal antibody B1C1 (Nilheden, Jeansson and Vahlne 1983; Huemer *et al.*, 1989), diluted 1:100 in PBS–Tween 20, supplemented with 1% bovine serum albumin, were added. The plates were incubated for 60 min after which the washing procedure was repeated. To each well was added 100 μl of alkaline phosphatase-conjugated, Fab fragment, goat antimouse IgG (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.), at a dilution of 1:1000. Finally, after 60 min at 37°C, the plates were washed again and the substrate *p*-nitrophenyl phosphate (Sigma P 104, Sigma Chemical Company, St Louis, MO, U.S.A.), dissolved in 200 μl aqueous diethanolamine solution (Fluka Chemie AG, Buchs, Switzerland), was added. Adsorption was read repeatedly in a Biotek microplate reader at wave lengths of 405 and 540 nm. Adsorption curves were then plotted at the time of attachment.

HSV production assay

GMK cells were inoculated with 0.5 ml of virus suspension at an MOI of one PFU per cell. Virus was allowed to adsorb for 1 h at room temperature. The cells were then washed three times with Eagle's MEM, reincubated at 37°C for 24 h with 3 ml of maintenance medium, with and without the addition of test substances (Tables 4 and 5). The dishes were frozen and thawed rapidly in three consecutive cycles, after which the cells and the medium were transferred to a centrifuge tube and centrifuged for 10 min at 1000 rev/mins to remove cell debris. Plaque titration

Table 3. Effects of tobacco chemicals on cellular protein production and cell growth on GMK cells

Substance	Concentration (mg/ml)	mg proteins /culture (mean ± SE)	Millions of cells /culture (mean ± SE)
Nicotine	1.0	0.15 ± 0.09*	1.20 ± 0.26*
	0.5	0.23 ± 0.01	1.41 ± 0.20
	0.1	0.26 ± 0.01	1.95 ± 0.06
Anabasine	1.0	0.04 ± 0.01*	0.32 ± 0.14*
	0.1	0.19 ± 0.01*	1.67 ± 0.05
	0.01	0.27 ± 0.02	1.97 ± 0.05
DEN	1.0	0.10 ± 0.05*	1.50 ± 0.42*
	0.1	0.28 ± 0.02	1.98 ± 0.02
NNN	1.0	0.15 ± 0.02*	1.54 ± 0.04*
	0.1	0.27 ± 0.02	1.64 ± 0.05*
NNK	1.0	0.12 ± 0.00*	1.56 ± 0.04
	0.1	0.19 ± 0.01*	2.12 ± 0.02
Control		0.29 ± 0.01	2.10 ± 0.16

*Denotes significant difference compared with control.

Table 4. Effect of snuff extract at different concentrations on HSV production and at different MOIs

Dilution	MOI (PFU/cell)	Produced HSV (log PFU/culture, mean ± SE)	*n*
1:5	1	3.26 ± 0.15*	8
1:25	1	5.67 ± 0.26*	8
1:10	0.1	5.47 ± 0.27*	8
1:10	1	5.18 ± 0.29*	8
1:10	10	5.24 ± 0.02*	8
1:10	30	6.47 ± 0.29	8
Control	0.1	6.21 ± 0.20	8
Control	1	6.61 ± 0.29	8
Control	10	6.31 ± 0.26	8
Control	30	6.72 ± 0.12	8

*Denotes significant difference compared with control.

was then done to measure the progeny virus production (Vahlne *et al.*, 1981). Five-hundred μl of the supernatant, diluted from 10^{-1} to 10^{-6}, were seeded onto GMK cultures in duplicate. After 30 min the cultures were covered with plaquing medium, containing 1% methylcellulose, 2% fetal calf serum, penicillin, and streptomycin of the same concentrations as in the maintenance medium. Plaques were counted after 5 days.

In another set of experiments GMK cell monolayers were infected with HSV-1 at MOIs 0.1, 1.0, 10, and 30 PFU per cell. Virus was allowed to adsorb for 1 h at room temperature. The dishes were then rinsed three times with Eagle's MEM and reincubated for 24 h with snuff extract diluted 1:10. The cells were then harvested and assessed for progeny virus as described above.

Assay of HSV protein synthesis

Confluent HEp-2 cells in 5 cm Petri dishes were inoculated with HSV at an MOI of 20 PFU per cell. After 1 h of incubation at room temperature, cultures were rinsed three times with Eagle's MEM and exposed to snuff extracts and chemicals at the concen-

Table 5. Effect of tobacco chemicals on HSV-1 replication

Substance	Concentration mg/ml	Produced HSV/culture (log PFU mean ± SE)	Reduction (%)
Nicotine	1.0	4.52 ± 0.26*	>99
	0.5	5.41 ± 0.20*	92
	0.1	6.64 ± 0.21	
Anabasine	1.0	3.03 ± 0.61*	>99
	0.1	6.03 ± 0.20	68
	0.01	5.96 ± 0.09	63
	0.001	6.06 ± 0.12	66
NNN	1.0	5.14 ± 0.71*	96
	0.1	6.26 ± 0.24	46
	0.01	6.24 ± 0.56	48
NNK	1.0	5.02 ± 0.69*	97
	0.1	6.40 ± 0.31	25
	0.01	6.22 ± 0.57	50
DEN	1.0	3.08 ± 0.07*	>99
	0.1	7.01 ± 0.18	
	0.01	7.04 ± 0.22	
Control		6.52 ± 0.18	

Cultures were inoculated at a MOI of 1 PFU/cell.
*Denotes significant difference compared with controls.

trations stated above. For the labelling of immediate early proteins (α-proteins), cultures were incubated with [^{35}S]-methionine, approx. 25 μCi/culture and dissolved in methionine-free Iscoves medium (3 ml/culture) containing snuff extracts or tobacco chemicals from 1 to 4 h post-infection. To study the production of early proteins (β-proteins), the infected cells were incubated for 3 h in 5% CO_2 at 37°C in maintenance medium, with snuff extracts and tobacco chemical added as stated above. After this the cultures were rinsed and labelled in the same way as for α-proteins from 4 to 8 h post-infection. For labelling late proteins (γ-proteins), infected cultures were incubated for 7 h in 5% CO_2 at 37°C in maintenance medium with snuff extract and tobacco chemicals added, and labelled from 8 to 12 h post-infection, as described above. At the end of the labelling period the cultures were rinsed three times with ice-cold PBS to end amino acid incorporation, harvested with a rubber plunger, dissolved in a small volume of PBS and centrifuged for 4 min at 3000 rev/min in an Eppendorf centrifuge. The labelled cells were denatured and solubilized by heating for 3 min at 80°C in a small volume of 2% SDS, 5′ β-mercaptoethanol, and 0.05 M tris–hydroxychloride (pH 7.0). Fifty μl of the solubilized material from each culture were added to each well of the gel. In parallel, 50 μl of each sample were precipitated onto filter papers with 10% ice-cold TCA. The precipitate was washed twice with 6% TCA, once with ethanol/ether mixed in 50:50 proportions and once with ether only. The radioactivity of the TCA precipitates on dried filter papers was assessed by liquid scintillation.

PAGE was done as described by Morse *et al.* (1978) in a discontinuous buffer system, containing 0.1% SDS. The stacking and separation gel contained 3 and 9% acrylamide, respectively and were cross-linked with the *N,N′*-diallyltartardiamide (2.6% of acrylamide weight). All chemicals for gel preparation were purchased from Bio-Rad, Richmond, CA, U.S.A. The separation gel was 15 cm in length. Proteins used for molecular-weight calibration were [^{14}C]-methylated myosin, phosphorylase-b, bovine serum albumin, ovalbumin, carbonic anhydrase and lysozyme (Amersham International, Amersham, U.K.), with molecular weights of 200,000, 97,400, 69,000, 46,000, 30,000 and 14,300, respectively. Phosphorylase-b, however, splits and shows up in gel as two bands of mol. wt 100,000 and 92,500. Protein bands were designated according to Morse *et al.* (1978). Adsorbance measurements of the autoradiographic images were done in a Shimadzu CS 910 spectrophotometer, equipped with a CR 1B chromatoscan. The amount of each protein was recorded and calculated as a percentage of total adsorbance in the lane, as well as the percentage of TCA-precipitable radioactivity added to the lane. The overall relation of each individual protein between the variously treated cultures did not differ, no matter which of these methods was used. The data presented in Text Figs 1 and 2 are calculated from six different experiments.

Synthesis of viral thymidine kinase and DNA polymerase

To assay the production of viral thymidine kinase and DNA polymerase in infected cells, bioassays were used to measure virus-specific enzyme activity, which directly reflects produced amounts of these enzymes. Cultures were infected with HSV at a MOI of 1 PFU/cell. Virus was adsorbed for 1 h at room temperature after which cells were rinsed three times with Eagle's MEM and incubated in 5% CO_2 at 37°C, in maintenance medium containing snuff extract and tobacco chemicals, as stated in Text Figs 3(A) and 3(B). After 1, 4, 7 and 11 h, duplicate cultures were rinsed three times with ice-cold PBS, scraped off with a rubber plunger, dissolved in 1 ml PBS and frozen at −70°C. Enzyme assays for the determination of the DNA polymerase and thymidine kinase activities were as described earlier (Gronowitz *et al.*, 1984; Neumüller *et al.*, 1990) on samples diluted to 1:100. Assay time for DNA polymerase was 180 min, and the total amount of radioactivity available was 170,000 c.p.m.

Statistical methods

The results are presented as means ± SE. The statistical significance of differences was calculated by means of Student's *t*-test. $p < 0.05$ was regarded as statistically significant (Colton, 1974).

RESULTS

Evaluation of cell toxicity

Confluent GMK cell cultures were exposed to snuff extract in 1:1, 1:5 and 1:25 dilutions for 6 days. Light microscopic morphological changes interpreted as signs of toxicity could be detected after 4 days in cultures exposed to undiluted snuff extract; after 6 days, slight signs of toxicity were observed also in cultures exposed to snuff extract at dilution 1:5. However, no morphological signs of toxicity were detected in cells exposed to snuff extract at dilution 1:25. Clear morphological signs of toxicity were found after 3 days in GMK cultures exposed to 1.0 mg/ml of nicotine, anabasine and DEN. Slight toxic effects were also observed in cultures exposed to 1.0 mg/ml of NNN and NNK after 3 days and clearly after 6 days. But no morphological signs of toxicity could be detected in cultures exposed to 0.1 mg/ml of the drugs used.

Cell counts and quantitative analyses of soluble cellular proteins are presented as averages of three cultures (Tables 2 and 3). Snuff extract diluted 1:5 exerted a 33% decrease in cell growth and 50% decrease of cellular protein production. In contrast, snuff extract diluted 1:25 stimulated cell growth, yielding a 13% increase in cell number, although no increase of protein synthesis was found. The growth rate of GMK cells was affected by nicotine, anabasine, NNN, NNK and DEN at the concentration of 1 mg/ml, and for NNN at a concentration of 0.1 mg/ml. Cellular protein synthesis was affected by anabasine at 0.1 mg/ml, by DEN at 1.0 mg/ml, by NNN at 1.0 mg/ml, and by NNK at 0.1 mg/ml.

Effect of snuff extract, nicotine, anabasine, DEN, NNN and NNK on HSV attachment to cells

The kinetics of the attachment of HSV to cellular receptors were studied in the presence of snuff extracts, nicotine, anabasine, DEN, NNN and NNK.

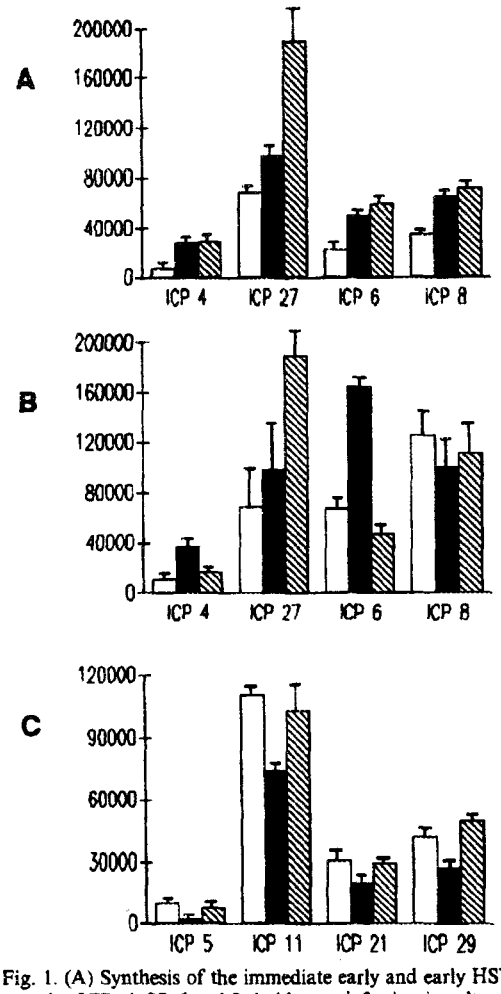

Fig. 1. (A) Synthesis of the immediate early and early HSV proteins ICPs 4, 27, 6 and 8, 1–4 h post-infection in cultures exposed to snuff extract at dilution 1:5 and 1:25 and controls which were not exposed to Eagle's MEM only. (B) Synthesis of ICPs 4, 27, 6 and 8, 4–8 h post infection in cultures exposed to snuff and controls. (C) Synthesis of the late HSV proteins ICPs 5, 11, 21 and 29 in HSV-infected cultures exposed to snuff and controls. Snuff diluted 1:5 (solid bars), snuff diluted 1:25 (hatched bars), and controls (open bars). Values indicate the densitometric adsorbance obtained in the autoradiographic images from six different experiments (mean ± SE).

After intervals ranging from 0 to 120 min, the amount of cell-associated virus was determined. Snuff extract diluted 1:2 completely inhibited attachment and a dilution of 1:5 also affected attachment. Nicotine at a concentration of 1.0 mg/ml, but not at 0.5 mg/ml, had a slight effect on HSV-1 attachment. Neither NNN, NNK, DEN nor anabasine had any effect on the attachment of HSV to cellular receptors at any of the concentrations tested (1.0 mg/ml and less).

Effect of tobacco extracts, nicotine, anabasine, DEN, NNN and NNK on HSV replication in GMK cells

Snuff extract effectively reduced the production of progeny virus (Table 4). The effect was related to both the concentration of snuff extract and the multiplicity of the infection. In GMK cell cultures inoculated with HSV-1 at a MOI of 1 PFU/cell, snuff extract diluted 1:5 reduced the HSV-1 production more than three log units (99.9%), whereas snuff extract diluted 1:25 exerted a one log unit (90%) reduction of progeny virus, as compared with the controls. The reductions induced by snuff extract at dilution of 1:5 and 1:25 were statistically significant. The inhibiting effect on HSV replication of the snuff extract diluted 1:10 was pronounced at MOIs of 0.1, 1.0 and 10 PFU/cell. However, at an MOI of 30 PFU/cell, the inhibitory effect of the snuff extract seemed to be partially overcome.

Nicotine at concentrations 1.0 and 0.5 mg/ml significantly reduced the production of HSV progeny (Table 5). Anabasine at a concentration of 1.0 mg/ml, and DEN at 1.0 mg/ml also induced a significant reduction of HSV progeny. The effects of HSV production induced by nicotine, anabasine and DEN at lower concentrations were not statistically significant, neither were the effects of NNN and NNK at non-toxic concentrations.

Protein synthesis

In the presence of snuff extract an increase of immediate early HSV proteins was found [Plate Fig. 4 and Text Figs 1(A) and (B)]. At both labelling intervals—1–4 h and 4–8 h post-infection—snuff extract induced a significant increase in the amount of ICP 4 in infected cells. This increase was most pronounced at the higher concentration of tobacco

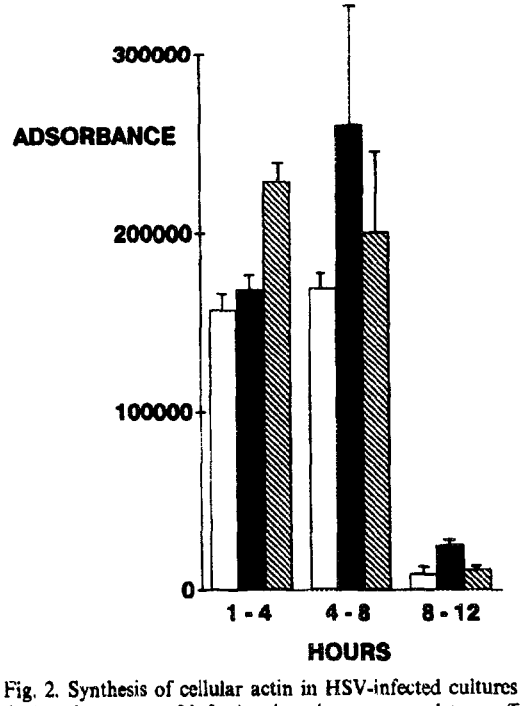

Fig. 2. Synthesis of cellular actin in HSV-infected cultures during the course of infection in cultures exposed to snuff extracts diluted 1:5 (solid bars) and 1:25 (hatched bars), as well as in controls exposed to Eagle's MEM only (open bars). Synthesis was measured as adsorbance in the autoradiographic images of the SDS–PAGE from six different experiments (mean ± SE).

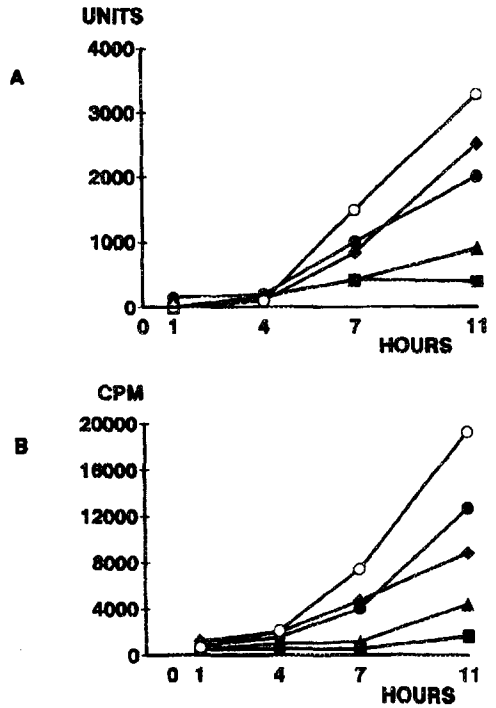

Fig. 3. Production of viral thymidine kinase (A) and DNA polymerase (B) measured as activity of these enzymes in HSV-infected cultures 1–11 h post-infection Activity of thymidine kinase was calculated in units as described by Gronowitz *et al.* (1984) and DNA polymerase activity as counts per min. Cultures were exposed to snuff extract at dilution 1:5 (■) and 1:25 (▲), nicotine 1.0 mg/ml (◆), anabasine 1.0 mg/ml (●), and controls with Eagle's MEM only (○).

extract. More of ICP 27 was also obtained by the addition of snuff extract to the cultures but the increase in quantity of ICP 27 was initially more by snuff at a dilution of 1:25 than of 1:5. The difference was significant only at the lower of these concentrations. During the first two labelling intervals, the relative amount of the early gene product ICP 6 was increased in the presence of the tobacco extract [Plate Fig. 4, and Text Figs 1(A) and (B)]. This increase was significant from 4 to 8 h post-infection. Nicotine at the relatively toxic concentration of 0.5 mg/ml had similar effect on the amounts of ICPs 4, 27 and 6 during the first labelling period. During later labelling periods, nicotine at this concentration induced a general decrease of all HSV proteins.

The production of γ-proteins, measured at 8–12 h post-infection, was reduced by addition of snuff extract at a dilution of 1:5 to HSV infected cultures [Text Fig. 1(C)]. The reduction of ICP 5, ICP 11 and ICP 29 production induced by snuff diluted 1:5 was significant. No statistically significant effects could be seen on the production of ICPs 5, 11 (gB), 21 (gE) and 29 in HSV infected cultures exposed to snuff extract at dilution of 1:25. Neither was the production of ICP 21 significantly affected at a dilution of 1:5 [Text Fig. 2(c)]. The production of γ-proteins increased during the course of infection in control cultures but in cultures exposed to snuff the increase in production of γ-proteins was markedly reduced. The relative reduction in γ-protein production in snuff exposed cultures could still be seen when followed up to 24 h post-infection. Between 20 and 24 h post-infection the production of ICPs 5, 11 and 29 was only 28, 55 and 63% of the production of these proteins in HSV-infected control cultures. These differences were significant. Anabasine, DEN, NNN and NNK had no significant effect on HSV protein synthesis when tested at non-toxic concentrations.

Production of thymidine kinase and DNA polymerase

The activities of the HSV-induced DNA polymerase and thymidine kinase were studied with bioassays as an indicator of the production of these two β-proteins. The activity of these viral enzymes, in relation to the course of infection is plotted in Text Fig. 3. The activity of thymidine kinase increased in control cultures from 1 to 11 h post-infection. Eleven h post-infection snuff extract at a dilution of 1:5 caused an 88% decrease in activity of thymidine kinase, and in the presence of snuff extract at a dilution of 1:25 there was a reduction of 72% [Text Fig. 3(A)]. The activity of DNA polymerase increased from 1 to 11 h post-infection but to a lesser extent in the cultures exposed to snuff and tobacco chemicals. The reduction exerted by snuff extracts remained during the course of infection. Activity of DNA polymerase was reduced by 97% by snuff extract at a dilution of 1:5 and by 77% by snuff extract at a dilution of 1:25 [Text Fig. 3(B)]. This reduction was significant for both concentrations tested at 11 h post-infection.

Nicotine and anabasine at a concentration of 1.0 mg/ml exerted a 23 and 39% decrease in thymidine kinase activity and depressed the activity of DNA polymerase by 34 and 54%, respectively, when measured 11 h post-infection. At lower, non-toxic concentrations of these drugs no effects on activity of thymidine kinase and DNA polymerase were observed. DEN, NNN and NNK had no effects on thymidine kinase or DNA polymerase at the concentrations tested.

Plate 1

Fig. 4. The autoradiographic image of HSV-1 proteins electrophoretically separated in a 9.25% polyacrylamide gel. Proteins from infected cells labelled with [^{35}S]-methionine 1–4 h post-infection (lane 1–3), 4–8 h post-infection (lane 4–6) and 8–12 h post-infection (lane 7–9). Molecular-weight markers are shown in lane M. Lane 1, 4 and 7 are from control cultures with Eagle's MEM only. Lane 2, 5 and 8 are from cultures exposed to snuff extract at dilution 1:5 and lane 3, 6 and 9 are from cultures exposed to snuff extracts at dilution 1:25. The numbers on the left indicate the different ICPs according to Morse *et al.* (1978).

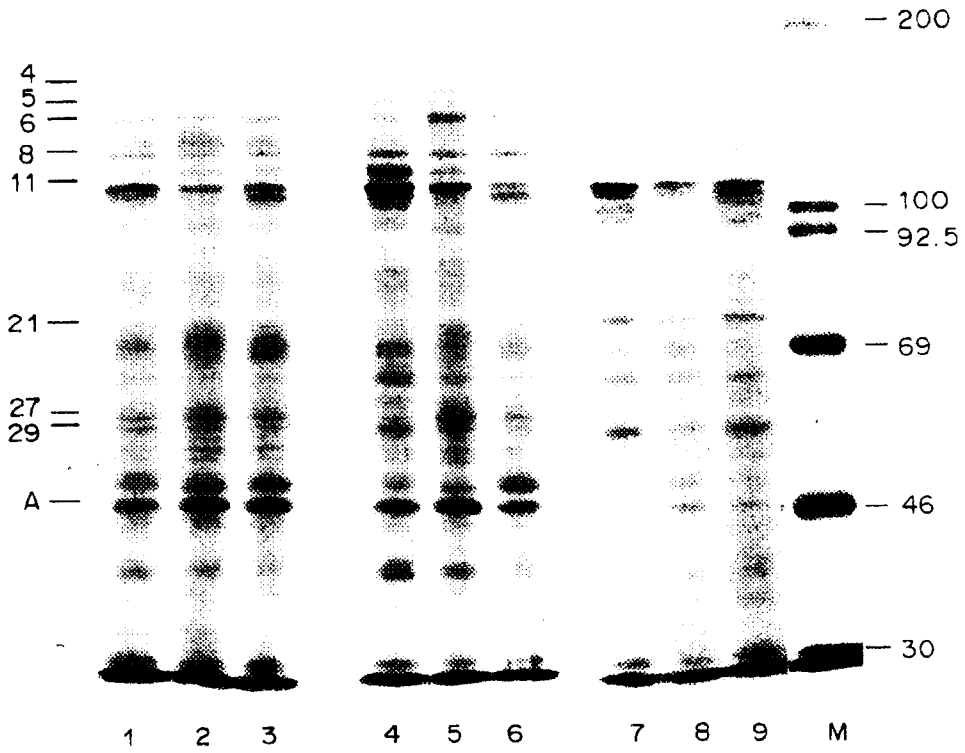

Plate 1

Production of actin in HSV infected cells

The autoradiographic images of SDS–PAGE revealed that the decrease in production of the cellular protein actin 4 h post-infection, and later in HSV-infected cells, was significantly affected by the addition of snuff extracts to the maintenance medium [Text Fig. 2 and Plate Fig. 4]. From 4 to 8 h post-infection, the relative synthesis of actin was 55% higher in cultures exposed to snuff extract at a dilution of 1:5 than in control cultures. In cultures exposed to snuff extracts at a dilution of 1:25, actin production from 4 to 8 h was slightly but not significantly higher than in control cultures. After 8 h the production of actin almost disappeared in control cultures, but constituted 7.5% of the total protein synthesis in cultures exposed to snuff extract at a dilution of 1:5 and 2.2% at a dilution of 1:25. No significant effect on actin production in HSV-infected cultures were observed when non-toxic doses of nicotine, anabasine, DEN, NNN or NNK were added.

DISCUSSION

Aqueous extract of snuff inhibits HSV replication in a dose-dependent manner (Hirsch *et al.*, 1984b; Oh *et al.*, 1990), a finding that was confirmed here. The inhibitory effect of snuff extract on HSV attachment to cells (Hirsch *et al.*, 1984b) could not be attributed to any of the tobacco chemicals we studied. However, in all our experiments on HSV replication and virus protein synthesis, snuff extract or tobacco chemicals were added 1 h post-infection in order not to interfere with the HSV adsorption to the cells.

Although our more extensive investigation of cell toxicity revealed that snuff extract, even at a dilution of 1:5, exerted a toxic effect on the cell growth and cellular protein synthesis *in vitro*, it had no significant toxic effects at lower concentrations. This suggests that the earlier reported effect on HSV replication (Hirsch *et al.*, 1984b; Stich *et al.*, 1987; Oh *et al.*, 1989) is probably a specific interaction between viral replication and tobacco chemicals.

Nicotine and anabasine are the water-soluble alkaloids found at the highest concentrations in both American and Swedish snuff. The tobacco-specific nitrosamines NNN and NNK are two well-known carcinogens in snuff and are present in the saliva of snuff dippers (Hoffmann and Adams, 1981; Hoffmann and Hecht, 1985). None of these substances had any significant effect on the replication of HSV when administered in non-toxic doses. Furthermore, the concentrations of NNN and NNK in the snuff extract were far below the levels at which they are toxic to cells or interfere with viral replication. DEN is another nitrosamine present in significant amounts in American snuff, but only occasionally in Swedish snuff (IARC, 1985). DEN has earlier been shown to inhibit HSV replication *in vitro* at a concentration of 1.0 mg/ml (Roane, 1978). The rationale for studying DEN was to compare its effect on HSV replication with those of NNN and NNK. The inhibitory effect of 1 mg/ml of DEN on HSV replication was confirmed. However, at this concentration it was found to be toxic to GMK cells.

Nicotine was toxic to GMK cells at a concentration of 1.0 mg/ml, slightly toxic at a concentration of 0.5 mg/ml but non-toxic at a concentration of 0.1 mg/ml. The inhibitory effect on HSV replication of nicotine at 0.5 mg/ml was significant but at a concentration of 0.1 mg/ml no inhibitory effect was obtained. Anabasine, an alkaloid related to nicotine and present in significant amounts in tobacco products, inhibited viral replication. However, this was only at concentrations toxic to cells, and the effect of anabasine at the concentrations found in snuff extracts was not statistically significant.

The inhibitory effect of tobacco extracts on HSV replication was more pronounced at low MOIs. The less inhibitory effect obtained at high MOIs supports the assumption that the effect of the snuff extract on HSV replication is exerted on viral functions and is not a secondary phenomenon due to cell toxic effects. Furthermore, at low MOIs the nitrosamines NNN, and NNK, as well as anabasine and nicotine, might have an effect on HSV replication not seen at the MOI (1 PFU/cell) studied. Nor can a possible synergistic effect of the tobacco chemicals be excluded.

The tobacco-induced inhibition of HSV replication reported appears early in the virus replication cycle, i.e. before or at the level of DNA replication (Hirsch *et al.*, 1984b; Stich *et al.*, 1987; Oh *et al.*, 1989, 1990). This conclusion is supported by the effects of snuff extract on HSV protein synthesis in our infected cells. The expression of the HSV genome in infected cells is sequentially regulated in five cascades of protein synthesis. The five groups of proteins are designated α-, β_1-, β_2-, γ_1- and γ_2-, of which α- and β-proteins are synthesized before progeny HSV DNA (Honess and Roizman, 1974; Wagner, 1984; Roizman and Sears, 1990). Synthesis of α-protein requires no prior protein synthesis but β-proteins require previous synthesis of α-proteins and in turn γ-proteins require previous β-protein synthesis for their production. An inhibition of β- and γ-protein synthesis also implies a prolonged production of α-proteins (Honess and Roizman, 1974; Faber and Wilcon, 1986). The increased production or accumulation of α-proteins and the decreased production of γ-proteins in the presence of tobacco extracts imply that there is a block in the infection between the production of these two groups of proteins. The increased production of ICP 6 and ICP 8 and the probably decreased production of thymidine kinase and DNA polymerase indicate that the block is before the synthesis of β_2-proteins but after β_1-proteins.

The mechanism of the block in virus replication is not clear but there are several possible explanations. One is that snuff extract blocks viral DNA replication. Stich *et al.* (1987) and Oh *et al.* (1989, 1990) found no effect on the synthesis of α- or β-protein when HSV-infected Vero cells were exposed to snuff extracts or smoked tobacco tar, but found a significant reduction of γ-protein synthesis. They concluded that the reduction of γ-protein synthesis was secondary to the suppression of DNA synthesis. Our results disprove that tobacco chemicals act solely at the level of DNA synthesis in HSV replication.

Another possible mechanism is that snuff extract interferes with the virion host shut-off function. The finding of prolonged synthesis of ICP 4 and ICP 27

and of cellular actin in the infected cells exposed to snuff extract supports this. The HSV host shut-off can be divided into two phases; a primary phase is mediated by a virion component that does not require synthesis of viral DNA and a late phase reduces the remaining levels of host protein synthesis (Kwong, Kruper and Frenkel, 1988; Read and Frenkel, 1983; Fenwick and Clark, 1982). The latter requires expression of viral genes. Both phases are reported to be coded by the same gene, mapping between 0.602 and 0.606 and encoding for a 58 kDa protein. The finding that giving snuff extract induces a prolonged synthesis of cellular actin in HSV-infected cells also indicates that tobacco chemicals might help cells survive a herpetic infection, despite the toxic effects of the chemicals. This may be of importance for the carcinogenic effects of the combination of tobacco and HSV observed in laboratory animals (Hirsch, Johansson and Vahlne, 1984a; Larsson *et al.*, 1989; Park *et al.*, 1986).

A third possible mechanism could be that substances in snuff extract interact with α-proteins. The characterization of temperature-sensitive and deletion mutants has revealed that ICP 4 and ICP 27, and in resting cells ICP 22, are essential for a productive HSV infection, and the functional and physical properties of these have also been extensively investigated (DeLuca and Schaffer, 1988; Rice and Knipe, 1988; Sears *et al.*, 1985). The α-proteins affect the transcription of specific subsets of viral genes. β-protein synthesis is dependent only on the expression of functional α-proteins, whereas the γ-proteins also require various degrees of viral DNA synthesis for their production (Honess and Roizman, 1974). After synthesis ICP 4 rapidly localizes to the nucleus. It is likely that ICP 4 initially localizes to sites in the nucleus defined by cellular structures and later localizes to intranuclear structures assembled as viral DNA replication proceeds (Randall and Dinwoodie, 1986; Knipe *et al.*, 1987). How ICP 4 is transported from the cytoplasm to the nucleus and localized there is not known. A possible effect of the substances in tobacco could be interference with ICP 4, which would lead to a failure in the autoregulative mechanisms of the ICP 4 synthesis (DeLuca and Schaffer, 1988).

ICP 4 and ICP 27 are potent trans-acting transcriptional activators (Arsenakis *et al.*, 1986). ICP 4 is also a potent activator of HIV replication, a potent amplifier of the rabbit β-globulin gene, and of bovine papilloma virus DNA in human cells; thus, it has an effect also on alien DNA (Albrecht *et al.*, 1989; Schlehofer, Ehrbar and zur Hausen, 1986; Smiley, Smibert and Everett, 1987). We have recently been able to show that patients with squamous-cell carcinomas of the head and neck region more frequently have antibodies to ICP 4 than HSV-positive control patients (Larsson *et al.*, 1991).

Our findings give further insight into the inhibitory effect of non-smoked tobacco on cytolytic HSV infection. The relevance of this for the earlier reported carcinogenic effects of snuff and HSV requires further investigation.

Acknowledgements—We are grateful to Dr Klaus Brunnemann, American Health Foundation, New York and Dr B-G Österdahl, Swedish Food Administration, Uppsala, Sweden, for the analyses of TSNA in snuff extract and to Dr D. Hoffmann American Health Foundation, New York, for providing NNN and NNK. This study was supported by grants from Göteborg Medical Society, Grant No. 90/196, Svenska Tobaks AB, Grant No. 8724, and Swedish Cancer Society, Grant No. 2555.

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