Archs oral Biol. Vol. 32, No. 4, pp. 291–296, 1987
Printed in Great Britain.

0003-9969/87 $3.00 + 0.00

EFFECT OF SMOKELESS TOBACCO ON THE REPLICATION OF HERPES SIMPLEX VIRUS *IN VITRO* AND ON PRODUCTION OF VIRAL LESIONS IN HAMSTER CHEEK POUCH

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Summary—Previous experiments have shown that combination of herpes simplex virus (HSV) infection and simulated snuff-dipping in hamster buccal pouches enhances the development of micro-invasive squamous cell carcinoma in cheek pouch epithelium. The effect has now been determined of water-extractable components of snuff (snuff-extract) on the growth and the cell-lysing activity of HSV. Various dilutions of snuff-extract in tissue culture medium significantly inhibited the growth of HSV in Vero cell monolayers by inhibiting the viral DNA replication. Moreover, HSV was inactivated and its cell-lysing activity lost when it was incubated with snuff-extract in cell-free condition. Snuff also had a similar anti-herpetic effect *in vivo*: HSV infection of pouch tissues followed by simulated snuff-dipping resulted in significant inhibition of viral growth. Thus snuff interferes with the DNA synthesis and cytolytic activity of HSV *in vitro* and *in vivo*, and this in turn, may increase its oncogenic capacity.

INTRODUCTION

Oral cancer appears to be associated with increased immune response to herpes simplex virus type 1 (HSV-1; Smith *et al.*, 1976; Shillitoe *et al.*, 1982). Several epidemiologic investigations indicate involvement of type 2 HSV (HSV-2) in the development of cancer in the uterine cervix and the vulva (Naib, Nahmias and Josey, 1966; Nahmias *et al.*, 1970; Rawls, Tompkins and Melnick, 1969; Rawls *et al.*, 1976). Furthermore, the viral antigens are expressed in tumour cells (Kaufman *et al.*, 1981), and both viral RNA (McDougall, Galloway and Fenoglio, 1980; McDougall *et al.*, 1982) and DNA (Frenkel *et al.*, 1972; Galloway and McDougall, 1983) are present in some cancer tissues from the uterine cervix. Malignant transformation of cells has been experimentally induced by ultraviolet-irradiated HSV (Duff and Rapp, 1971), fragments of HSV DNA (Jariwalla, Aurelian and TS'O, 1980), and photodynamically inactivated HSV (Li, Jerkofsky and Rapp, 1975). Formalin- or ultraviolet-inactivated HSV have been reported to induce uterine cancer in mice (Wentz *et al.*, 1981).

Snuff-dipping, the placement and retention of powdered tobacco in the mucobuccal fold of the mouth has been associated, in some epidemiological studies, with an increased incidence of human oral and pharyngeal cancer (Peacock, Greenberg and Brawley, 1960; Pindborg *et al.*, 1980; Winn *et al.*, 1981). However, several experiments have shown that placement of snuff in the mouth simulated snuff-dipping in animals does not induce malignant changes (Shklar *et al.*, 1985; Park, Sapp and Herbosa, 1987). Therefore, other factors may contribute to the development of tobacco-related oral malignancies. Tobacco and HSV are synergistic in the development of precancerous lesions in mice (Park *et al.*, 1985). Furthermore, repeated HSV infection in combination with simulated snuff-dipping leads to the development of oral cancer in rats (Hirsch and Johansson, 1983) and of micro-invasive, squamous-cell carcinoma in hamster buccal pouch tissues (Park *et al.*, 1987) but neither HSV infection nor simulated snuff-dipping alone induce carcinoma. HSV must be inactivated and lose its cytolytic activity to release its oncogenic capacity (Duff and Rapp, 1971). Water-extractable components of snuff (snuff-extract) inhibit the replication and infectivity of HSV-1 (Hirsch, Johansson and Vahlne, 1984a). We have now examined the action of snuff-extract on both HSV-1 and HSV-2 replication *in vitro* and *in vivo*.

MATERIALS AND METHODS

Viruses and cell

HSV-1 (F-strain) and HSV-2 (G-strain) were obtained from the American Type Culture Collection (ATCC), Rockville, Maryland, U.S.A. The viruses were propagated in Vero cell monolayers and the viral titres were adjusted to 5.0×10^7 plaque-forming units per millilitre (PFU/ml). The stock virus was stored at −80°C until used. Vero cells purchased from the ATCC were grown in Eagle's minimum essential medium (E-MEM, The Media Center, UCLA Molecular Biology Institute, Los Angeles, California, U.S.A.) supplemented with 5 per cent fetal bovine serum (FBS). The monolayers were cultured at 37°C in a 5 per cent carbon-dioxide atmosphere.

Preparation of snuff-extracts

Ten grammes of a commercially available snuff (Copenhagen®, U.S. Tobacco Co., Franklin Park, Illinois, U.S.A.) were mixed with 100 ml of E-MEM supplemented with 5 per cent FBS and incubated at

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37°C for 2 h, centrifuged at 450 *g* for 10 min. The supernatant was ultracentrifuged in Beckman SW 27 rotor (Beckman Instruments, Fullerton, California, U.S.A.) at 13,000 *g* for 1 h. Lastly, it was sterilized by filtration through a Millipore filter (pore size, 0.45 μ) after pH adjustment to 7.4 with 0.1 M HCl. This sterile extract was designated as the original snuff-extract (10 per cent). It was further diluted with E-MEM supplemented with 5 per cent FBS to make 2.0, 1.0, 0.4, 0.2 and 0.1 per cent snuff-extracts.

Determination of cell growth

This was by measuring the cell numbers and the synthesis of protein and DNA. Confluent Vero cell monolayers in 100 mm Petri dishes were trypsinized and counted; the cells were suspended in E-MEM and 5×10^6 cells per plate were plated onto 60 mm Petri dishes. Four hours after plating the culture medium was replaced with fresh medium or snuff-extracts (0.1, 0.2, 0.4, 1.0 or 2.0 per cent). The number of cells was counted after 24 and 48 h incubation at 37°C. There were ten cultures in each group at each time. The cells were harvested after 0, 24 and 48 h of incubation, and centrifuged at 400 *g* for 10 min. The cell pellets were washed three times with phosphate-buffered saline (PBS) and treated with 0.1 M NaOH. The protein content of the pellet was determined by the method of Lowry *et al.* (1951). Cellular DNA synthesis was determined by growing the Vero cells in the presence of [^{3}H]-thymidine (23.0 Ci/mM, New England Nuclear, Boston, Massachusetts, U.S.A.). Four hours after plating the cells (5×10^6 cells/Petri dish), the culture medium was replaced with either E-MEM or snuff-extracts containing 1.0 μCi/ml of [^{3}H]-thymidine. After incubation at 37°C for 24 or 48 h cultures were washed three times with 5 per cent trichloroacetic acid (TCA). The washed precipitates were hydrolysed with 10 per cent TCA for 30 min at 70°C and the hydrolysates were centrifuged. The supernatants were then mixed with scintillation cocktail solution and the radioactivity was measured in a Beckman Liquid Scintillation Counter.

Determination of the effect of snuff-extract on viral growth

Confluent Vero cell monolayer cultures were inoculated with HSV at 5 PFU per cell. Virus was allowed to adsorb for 1 h at 37°C in a CO_2 incubator. The cells were washed twice with PBS and then fresh E-MEM, supplemented with 5 per cent FBS or snuff-extracts (0.1, 0.2, 0.4, 1.0 or 2.0 per cent), was added. After continuous incubation at 37°C for 24 h, the culture medium or snuff-extracts were removed and the cells gently washed twice with cold PBS. They were then frozen and thawed three times in PBS, collected, and centrifuged at 450 *g* for 5 min. The viral titres were then determined from the supernatant using a plaque assay technique (Rapp, 1963). The experiment was repeated four times and the average values were taken and compared by Student's *t*-test.

Determination of the effect of snuff-extract on viral infectivity

To determine the effect of snuff-extract (2.0 per cent) on the infectivity of HSV in cell-free condition, 1.0 ml of HSV-1 or HSV-2 with a titre of 1.0×10^7 PFU/ml was mixed with 1.0 ml of either E-MEM or 5 per cent snuff-extract. The mixtures were then incubated at 37°C for 0, 1, 2, 4 or 6 h. At the end of incubation, the mixtures were ultracentrifuged using a Beckman SW 27 rotor at 55,000 *g* for 2 h at 4°C to precipitate virus particles. Supernatants were removed and the viral pellets were resuspended in PBS; viral titres were determined from the resuspended pellets using the plaque assay technique (Rapp, 1963). Each group comprised ten samples.

Isolation and analysis of viral and cellular DNA

Confluent Vero cell monolayers in 100 mm Petri dishes were infected HSV at 10 PFU/cell. After adsorption for 1 h at 37°C, the viral inoculum was removed, and the cells were washed twice with PBS. E-MEM, supplemented with 5 per cent FBS or snuff-extracts, was then added to the cultures along with 1.00 μCi/ml of [^{3}H]-thymidine (23.0 Ci/mM; New England Nuclear, Boston, Massachusetts, U.S.A.) per dish. After incubation at 37°C for 24 h the cells were collected and centrifuged. The cell pellets were washed twice with PBS, gently resuspended in TE buffer (10 mM tris, 10 mM EDTA, pH 7.6) containing 0.4 per cent sodium dodecyl sulphate (SDS), and 4.0 mg of proteinase, and incubated at 37°C for 24 h. CsCl was then added for a final density of 1.7 g/cm^3. Isopycnic equilibrium CsCl density-gradient centrifugation was performed in a Beckman rotor 50 (Beckman Instruments, Fullerton, California, U.S.A.) at 125,000 *g* for 48 h at 20°C. Gradients were dripped from the bottom of each tube, and fractions were collected. A portion of each fraction was spotted on a GF/C filter disk (Whatman, England), washed twice in cold 5 per cent TCA and once in cold distilled water, then dried at 60°C. Radioactivity in the spotted GF/C filter was measured in a scintillation cocktail solution with a Beckman Liquid Scintillation Counter. Portions of a few fractions were spotted on a refractometer, and their density was checked.

Effect of simulated snuff-dipping on viral growth in vivo

Forty-eight golden Syrian hamsters (random-bred male, 6–7 week old) purchased from the Simonson Laboratory (Gilroy, California, U.S.A.) were used. They were divided into four groups of 12 and inoculated with either HSV-1 or HSV-2. The animals in groups 1 and 2 were inoculated with HSV-1 (5×10^5 PFU/pouch) and the groups 3 and 4 with HSV-2 (3×10^5 PFU/pouch). Under ketamine HCl anaesthesia (100 mg/kg of body weight, intramuscular injection, Parke-Davis, Morris Plains, New Jersey, U.S.A.), the right and left buccal pouches were scarified with a 26-gauge needle in a cross-hatched pattern over an area of 2.0 cm^2. HSV was applied to this area and the tissues rubbed gently with a Q-tip for 20 s. In groups 1 and 3, simulated snuff-dipping into both pouches was done. The snuff was inserted via a hollow plastic cylinder with a central rod that when compressed once deposits a reproducible amount. There was a consistent delivery of approx. 0.1 g of snuff to each pouch. The simu-

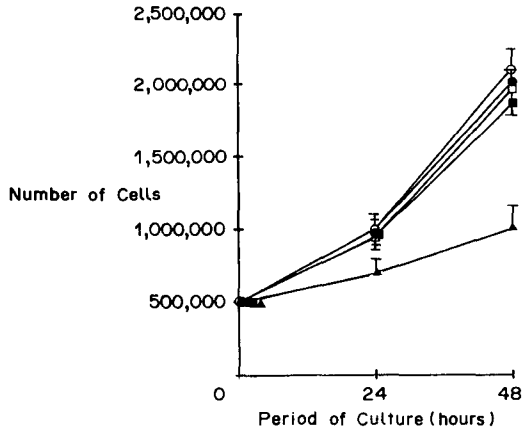

Fig. 1. Effect of snuff-extract on the growth of Vero cells; the vertical bar indicates one standard error of the mean. Control group, ●; 0.2 per cent snuff-extract, ○; 0.4 per cent snuff-extract, □; 1.0 per cent snuff-extract, ■; 2.0 per cent snuff-extract, ▲.

lated snuff-dipping was begun 2 h after the viral inoculation and continued twice daily for seven days. Three, five and seven days after the viral inoculation, four hamsters from each group were killed and their pouches excised, washed with PBS, weighed and finely minced with a scissors. The tissues were then homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, New York, U.S.A.) and made into a 3 per cent suspension of E-MEM supplement with 5 per cent FBS. The suspension was frozen and thawed three times, and centrifuged at 450 *g* for 10 min at 4°C. The supernatant was serially diluted the viral titres were assayed in monolayers of Vero cells by plaque assay (Rapp, 1963).

RESULTS

Effect of snuff-extracts on the growth of Vero cells

There was significant reduction in cell growth and protein synthesis after 48 h incubation with 2.0 per cent snuff-extract, but no significant inhibition with the more diluted snuff-extracts (Figs 1 and 2). Cellular DNA synthesis was also significantly inhibited in the presence of 2.0 per cent snuff-extract, but lower concentrations did not alter it (Fig. 3).

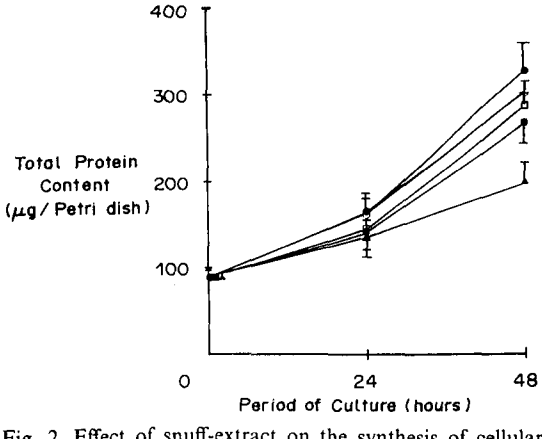

Fig. 2. Effect of snuff-extract on the synthesis of cellular protein; the vertical bar indicates one standard error of the mean. Control group, ●; 0.2 per cent snuff-extract, ○; 0.4 per cent snuff-extract, □; 10 per cent snuff-extract, ■; 2.0 per cent snuff-extract, ▲.

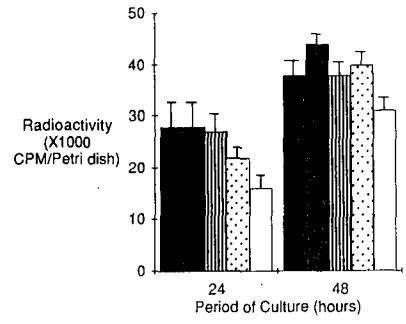

Fig. 3. Effect of snuff extract on the synthesis of cellular DNA; the vertical bar indicates one standard error of the mean. Control group, ■; 0.2 per cent snuff-extract, ▣; 0.4% per cent snuff-extract, ▥; 1.0 per cent snuff-extract, ⊡; 2.0 per cent snuff-extract □. CPM = counts/min.

Effect of snuff-extract on the in vitro *growth of HSV*

Snuff-extracts inhibited the growth of both HSV-1 and 2 in Vero cell monolayer cultures in a concentration-dependent fashion (Fig. 4): the higher the concentration of snuff-extract, the greater the inhibition. With 2.0 per cent snuff-extract, the viral yield was less than 3 per cent of that of control group. Furthermore, HSV-1 and 2 were more or less equally sensitive to the different concentrations of snuff-extract.

Effect of snuff-extract on the infectivity of HSV

In the control (E-MEM only) group, temperature inactivation of HSV-1 and 2 was obvious and by 6 h incubation at 37°C the infectivity was reduced by one log scale. Beyond this temperature inactivation, virus infectivity was significantly reduced by snuff-extract in a time-dependent fashion: the longer the incubation, the greater inhibition of infectivity (Fig. 5).

Effect of snuff-extract on HSV DNA synthesis

The amount of HSV DNA synthesized was significantly reduced in the presence of snuff-extracts in a concentration-dependent fashion (Figs 6 and 7): the higher the concentration, the greater the inhibition. The cell-culture DNA synthesis was also

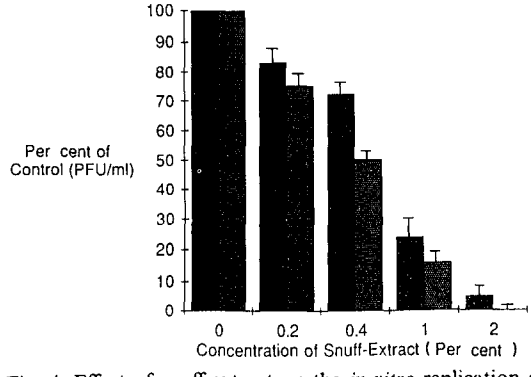

Fig. 4. Effect of snuff-extract on the *in vitro* replication of HSV-1 (■) and HSV-2 (▣). The vertical bar indicates one standard error of the mean.

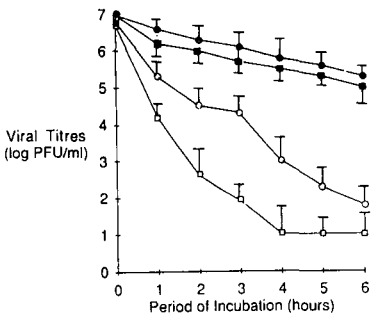

Fig. 5. Effect of snuff-extract on the infectivity of HSV-1 and HSV-2; the vertical bar indicates one standard error of the mean. HSV-1 control, ●; HSV-1 with snuff-extract, ○; HSV-2 control, ■; HSV-2 with snuff-extract, □.

diminished in the presence of 0.4, 1.0 or 2.0 per cent snuff-extract, but with 0.2 per cent snuff-extract, only the synthesis of HSV DNA was significantly inhibited.

Effect of simulated snuff-dipping on the development of HSV lesions and the in vivo *growth of HSV*

One day after inoculation with HSV-1 the pouches were erythematous; oedema appeared two days after inoculation, together with small vesicles and ulcers. All such changes were most prominent three or four days after inoculation and regressed rapidly thereafter. Seven days after there were no macroscopic lesions. In contrast, HSV-2 infection induced mild changes with minimal erythema and swelling and no ulceration. In animals receiving simulated snuff-

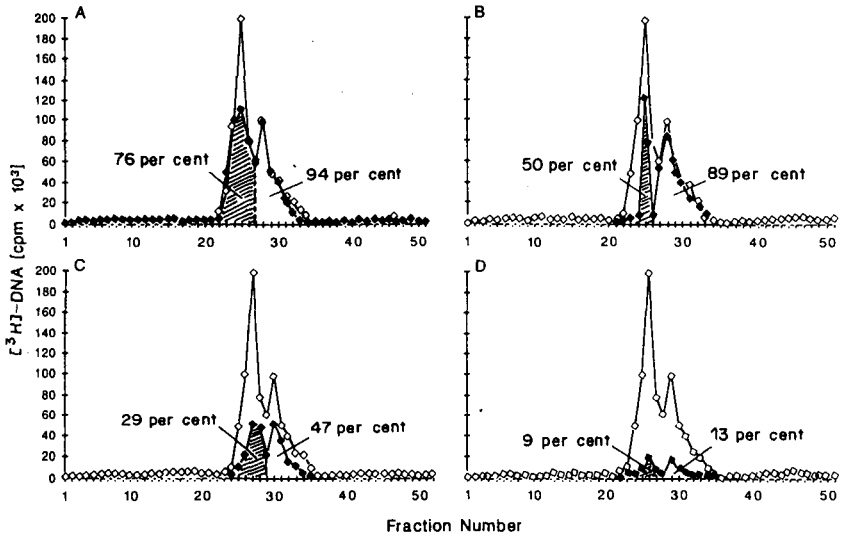

Fig. 6. Isopycnic equilibrium density gradients of DNA from HSV-1 infected Vero cells. The first peak with heavy density (1.72 g/cm³) represents the viral DNA and the second peak with lighter density (1.70 g/cm³) depicts the cellular DNA. The DNA synthesis was monitored in the absence (◇) or presence (◆) of snuff-extract (A, 0.2 per cent; B, 0.4 per cent; C, 1.0 per cent; D, 2.0 per cent). CPM = counts/min.

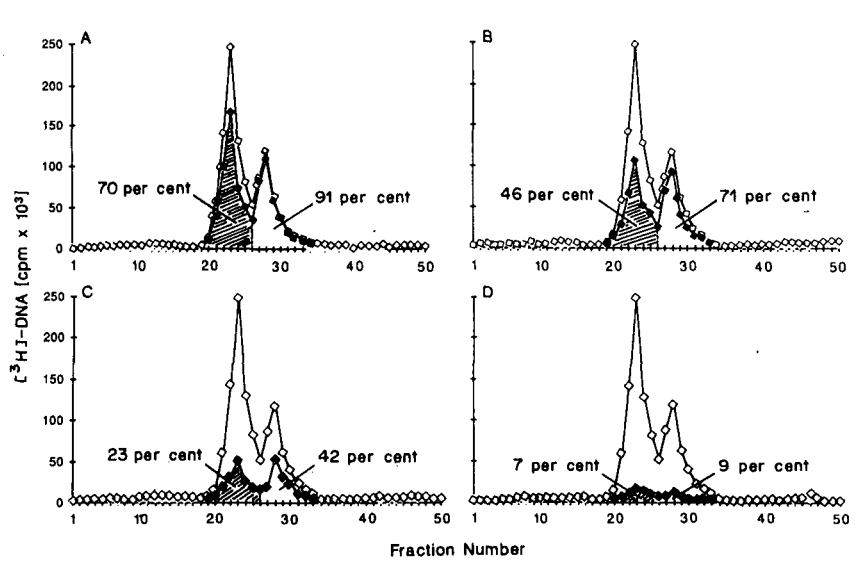

Fig. 7. Isopycnic equilibrium density gradients of DNA from HSV-2 infected Vero cells. The first peak with heavy density (1.72 g/cm³) depicts the viral DNA and the second peak with lighter density (1.70 g/cm³) is the cellular DNA. The DNA synthesis was determined in the absence (◇) or presence (◆) of snuff-extract (A, 0.2 per cent; B, 0.4 per cent; C, 1.0 per cent; D, 2.0 per cent). CPM = counts/min.

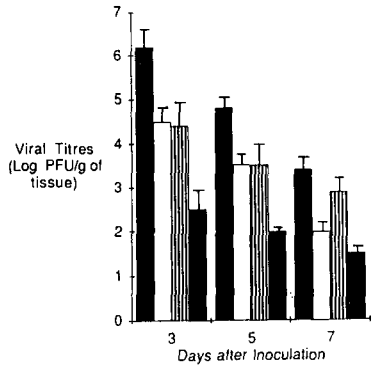

Fig. 8. Effect of simulated snuff-dipping to the hamster buccal pouches on the growth of HSV in pouch tissues; the vertical bar indicates one standard error of the mean. HSV-1 only, ■; HSV-1 and snuff-dipping, □; HSV-2 only, ▥; HSV-2; snuff-dipping, ▣.

dipping after HSV infection, HSV lesions were mild compared with the group infected with HSV alone. Vesicles and ulceration did not develop when snuff was placed in the pouches after HSV-1 infection. HSV-1 and HSV-2 were detectable in the mucosa three, five and seven days after inoculation and simulated snuff-dipping resulted in a significant reduction of HSV in pouch tissues (Fig. 8).

DISCUSSION

Snuff-extract inhibited the replication of HSV-1 and HSV-2 in Vero cell monolayers, confirming earlier findings with HSV-1 (Hirsch, Svennerholm and Vahlne, 1984b). In concentrations which did not interfere with cellular growth and the synthesis of cell protein and DNA, snuff-extracts selectively inhibited viral growth. However, at high concentrations snuff-extract markedly impaired the cellular metabolism. Highly concentrated snuff-extract also inactivated HSV in the cell-free condition and reduced its cytolytic activity. Simulated snuff-dipping not only inhibited the development of viral lesions in the hamster pouch, but also significantly suppressed the growth of HSV there.

To examine the mechanism of HSV inactivation by snuff-extract, its effect on the synthesis of cellular and viral DNA was determined in virus-infected cells. Again, at a concentration which did not interfere with the cellular DNA synthesis in HSV-infected cells, snuff-extract selectively inhibited viral DNA. However, higher concentrations of snuff-extract inhibited both the synthesis of cellular and viral DNA. Therefore, the *in-vitro* inhibition of HSV by snuff-extract may be due in part to its action on viral DNA synthesis. It is known that snuff-extract inhibits the adsorption process in HSV replication (Hirsch *et al.*, 1984b), but such effects cannot be responsible for the inhibition of viral DNA synthesis because the virus was allowed to adsorb to the cells in the absence of snuff-extract.

Snuff and its extract contain numerous substances with biological activity, so it is difficult to predict which ones are responsible for the inhibitory effect on HSV replication and cytolytic activity. Some chemical carcinogens, including nitrosamines, tobacco-specific *N*-nitrosamines (TSNA) and nicotine found in smoked tobacco tar or smokeless tobacco can inhibit the replication of HSV *in vitro* (Roane, 1978; Speelman *et al.*, 1981; Hirsch *et al.*, 1984b). Therefore, TSNA and nicotine from snuff, might be responsible for the inhibition in HSV replication and infectivity, as suggested by Hirsch *et al.* (1984b).

Acknowledgements—A part of this study was presented at the 64th General Session of the AADR at Washington, D.C., U.S.A., March 23, 1985, and was supported by a grant from the Smokeless Tobacco Research Council, Inc. No. 0052-R1 and NIH support RR05304.

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