A study on interactions of herpes simplex virus and tobacco with special reference to tumor development

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1990

Document Version:
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A STUDY ON INTERACTIONS OF HERPES SIMPLEX VIRUS AND TOBACCO - WITH SPECIAL REFERENCE TO TUMOR DEVELOPMENT

Per-Anders Larsson

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AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Göteborgs Universitet kommer att offentligen försvaras i föreläsningssalen, 2 tr., Institutionen för Klinisk Bakteriologi, Immunologi och Virologi, fredagen den 14 september, 1990, kl 09.00

Per-Anders Larsson.

Avhandlingen baseras på följande arbeten:

I Hirsch JM, Larsson P-A & Johansson SL. The reversibility of the snuff induced lesion: an experimental study in the rat. J. Oral Pathol. 1986: 15: 540-543.

II Johansson SL, Hirsch JM, Larsson P-A, Saidi J, & Österdahl BG. Snuff-induced carcinogenesis: effect of snuff in rats initiated with 4-nitroquinoline N-oxide. Cancer Research 1989: 49: 3063 - 3069.

III Larsson P-A, Johansson SL, Vahlne A & Hirsch JM. Snuff tumorigenesis: effects of long-term snuff administration after initiation with 4-nitroquinoline-N-oxide and herpes simplex virus type 1. J. Oral Pathol. Med. 1989: 18: 187-192.

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V Larsson P-A, Hirsch JM, Gronowitz JS & Vahlne A. Inhibition of herpes simplex virus replication and protein synthesis by non smoked tobacco, tobacco alkaloids and nitroseamines. Submitted for publication.

VI Larsson P-A, Edström S, Westin T, Nordkvist A, Hirsch JM. & Vahlne A. Tobacco consumption and antibodies against herpes simplex virus in patients with head and neck cancer. Submitted for publication.

ABSTRACT

Larsson, P-A., A STUDY ON INTERACTIONS OF HERPES SIMPLEX VIRUS AND TOBACCO - WITH SPECIAL REFERENCE TO TUMOR DEVELOPMENT
(page 1 to 44)

Department of Clinical Virology, University of Göteborg, Guldhedsgatan 10b, S-413 46 Göteborg, Sweden.

Thesis defended September 14, 1990.

Smoked and non-smoked tobacco as well as herpes simplex virus (HSV) are well known risk factors in the development of human cancer. It has earlier been shown that snuff extract and smoked tobacco tar block the replication of HSV at an early stage of the infectious cycle. This investigation was designed to study the interactions between tobacco and HSV with special reference to human cancer. Non-smoked tobacco induces lesions in rat oral mucosa which are reversible after a limited time of exposure. The investigation of possible tumor promoting effects of snuff in rats initiated with a subcarcinogenic dose of the oral carcinogen, 4-nitroquinoline N-oxide, showed that snuff has a weak carcinogenic effect but it does not solely act as a promoter in the tumor development. Exposition to the combination of HSV infection and tobacco results in a significant increase of tumor development in rats. This is not only due to the fact that inhibition of viral replication would allow HSV to express a possible carcinogenic effect since the treatment of HSV-infected mice with acyclovir does not increase the tumor incidence.

As studied in vitro, non-smoked tobacco extract interferes with the replicative cycle of HSV at an early level of the infection. Cellular functions, as monitored by actin synthesis, are protected in HSV infected cells in the presence of tobacco extracts. Immediate early (α-) and some early (β-) proteins are accumulated in HSV infected cells when exposed to non-smoked tobacco. A high frequency of antibody reactivity to the HSV α-protein ICP 4 was found in head and neck cancer patients, who are predominantly smokers.

The results of the present study shows that the combination of HSV and tobacco is tumorigenic. The accumulation of immediate early proteins observed in HSV infected cells when exposed to tobacco extracts may be of relevance to development of cancer in tobacco users.

Key words: Herpes simplex virus, tobacco, snuff, protein synthesis, infection, tumor, cancer.

ISBN: 91-628-0067-1

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To Elisabeth and Malin

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PREFACE

This thesis is based on the following papers which will be referred to in the text by their Roman numerals:

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ABBREVIATIONS

4-NQO	4 nitroquinoline N-oxide
BL	Burkitt's lymfoma
BSA	bovine serum albumin
CMV	cytomegalovirus
DEN	dietyl-N-nitrosamine
EBV	Epstein-Barr virus
ELISA	enzyme linked immuno sorbent assay
FCS	fetal calf serum
GMK	green monkey kidney - cells
HEp-2	human epidermoid carcinoma No.2 cell
HPLC	high performance liquid chromatography
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
ICP	infected cell protein
MEM	minimal essential medium
MOI	multiplicity of infection
NNK	N-(N-methyl-N-nitrosamino)-1-(3-pyrridyl)butanone
NNN	N-nitrosonornicotine
NPC	nasopharyngeal carcinoma
PAGE	polyacrylamide gel electrophoresis
PBS	$Na_2H_2PO_4$ buffered NaCl pH 7.4.
PFU	plaque forming units
SDS	sodium dodecyl sulphate
SE	standard error of the mean
TCA	trichloroacetic acid
TPA	12-O-tetradecanoylphorbol 13-acetate
TSNA	tobacco specific nitrosamines
VZV	varicellae zoster virus

INTRODUCTION

The use of tobacco is closely related to cancer in man (56, 115). Also the use of non-smoked tobacco, i.e. snuff dipping and tobacco chewing, has been shown to give an increased risk of cancer development (124). Experimentally, non-smoked tobacco has a week carcinogenic effect (45). Although strong carcinogens are present in tobacco, the specific effect of tobacco chemicals in tumor development has not been clarified, and a possible role of other factors must be considered. Herpes simplex virus (HSV) infection has also been implimented in human cancer (85, 100). Although there is a substantial experimental evidence that HSV can transform cells in vitro, the relevance of this for human cancer has been disputed (68). A prerequisite for HSV to transform cells is that the otherwise cytolytical replicative cycle of the virus is inhibited. Tobacco has been shown to inhibit HSV replication at an early level, i.e. before or at the DNA synthesis (52, 80, 110). Both smoking and HSV infection are related for an increased risk for cancer of the uterine cervix (122). Thus, both experimental and epidemiological studies suggest a possible interaction of tobacco and HSV in tumor development in man.

AIM OF THIS INVESTIGATION

The aim of this investigation is to contribute to the understanding of interactions between tobacco and herpes simplex virus and their possible role as risk factors in development of human cancer. Particular attention was given to the following:

Progression of the snuff induced lesion in rat oral mucosa after cessation of exposure to snuff.

Tumor promoting effects of snuff in rats initiated with a subcarcinogenic dose of a well known carcinogen.

Carcinogenic effects of HSV and snuff and the combination of these two components in rats.

Whether inhibition of the cytolytic replicative cycle of virus per se affects tumor incidence in HSV infected animals.

Effects of nicotine, anabasine, and the tobacco specific nitrosamines NNN and NNK on HSV-infection in vitro.

Effects of aqueos snuff extract on the production of HSV infected cell proteins in vitro, to assess the level of the block in HSV replication.

Seroprevalence and antibody profile to HSV proteins in patients with carcinomas of the head and neck region.

GENERAL BACKGROUND

Tobacco and cancer

In his report The Health Consequences of Smoking, 1982 the Surgeon General of the United States concludes that cigarette smoking is the major single cause of cancer mortality in the United States, that the contribution of tobacco to all cancer deaths is estimated to be 30 percent, and that 50 to 70 percent of oral and laryngeal cancer deaths are associated with smoking (115). These figures are applicable also in Sweden although the proportion of smokers in this country is slightly smaller than in the U.S. - approximately 26 per cent of the Swedish population are smokers (120). However, due to intensified health educational efforts cigarette consumption has declined during the last few years.

This is in stark contrast to the increase in the use of moist snuff in Western Europe and the United States during the last decade where it has become especially popular among young people (56, 79, 83, 92). Around 35 per cent of young males in Sweden are snuff dippers (83). Snuff dipping has, however, been a common habit for many decades in certain areas. The most studied snuff dipping cohorts are Swedish males and women in south eastern USA (56, 124).

Snuff dipping is often falsely portrayed as a less health threatening substitute for cigarette smoking, although epidemiological studies have shown considerable detrimental effects on both general health and and oral health. Earlier studies on general health effects of snuff dipping and tobacco chewing have shown that smokeless tobacco may affect the reproductive system (59) and the cardiovascular system (11). It has also been reported that a cohort of tobacco chewers age-adjusted death rate was significantly higher than in non chewing controls (44).

The application of snuff results in characteristic lesions of the oral mucous membrane at the site of the quid (7, 49, 82) and an increased risk of developing intraoral leukoplakias has been attributed to the use of unburned tobacco (71).

The most serious complication associated with snuff dipping is the markedly increased risk of developing oral cancer especially after long time exposure. The use of tobacco in an unsmoked form was linked with oral cavity cancer as early as 1915 in New York when Abbe 1915 reported a case of cancer of the cheek, occurring in a chronic snuff dipper (1).

Studies on the carcinogenic effects of snuff have, however, given controversial results. A prevalence survey of 15,500 snuff using patients in Tennessee yielded fewer than 2000 patients with any mucosal abnormality and only two malignancies (107). There might be two explanations of these results. One is that the carcinogenic effect of snuff is weak, which might be due to the presence of components in snuff inhibiting tumor induction (16, 45). The other is that, since oral cancer is rare, it is difficult to obtain significant results enabling adequate conclusions even in large populations (124). Studies during the last decade have provided more information on oral cancer and snuff dipping. Winn et al. demonstrated that snuff exposure lasting for 4 decades or longer was associated with approximately a 50 times increased risk of developing squamous cell carcinoma of the oral cavity (123, 124).

Wheather the typical snuff dipper's lesion is a precancerous state of oral epithelium (7, 49, 82) is still a controversial issue but cancers occuring in snuff users are more frequently associated with the presence of leukoplakia (15, 31, 104, 124). The prevalence in Sweden of leukoplakias has been estimated to be 3.6 per cent and the reported frequency of malignant transformation to 4 per cent within 20 years (7, 31)

The International Agency for Reasearch on Cancer and the National Institute of Health have stated that there is sufficient evidence to regard snuff as an oral carcinogenic agent when used as in North America and Western Europe (56, 79).

Digestive and respiratory tract cancers have also been linked with the use of smokeless tobacco, but the evidence is inconclusive. Only esophageal cancer has been examined to some extent, and in a study carried out in Puerto Rico positive associations between tobacco chewing and esophageal cancer was found both in men and women (69). Wynder and Bross found a positive history of tobacco chewing in 20 per cent of oesophageal cancers compared to 10 per cent in controls (127). However, all chewers were also smokers.

Tobacco contains at least 2549 chemical substances (27) and in snuff at least three types of known carcinogens have been identified. These are polycyclic aromatic

hydrocarbons, radioactive substances (of which ^{210}Po is the best known), and nitroseamines including some 20 tobacco specific, volatile and non-volatile nitroseamines. The tobacco specific nitroseamines (TSNA) occur in snuff in at least 100 times the quantities found in other consumer products (54). The TSNA N-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are regarded to be of importance for the carcinogenic properties of snuff and has been shown to be present in the saliva of snuff dippers (53). Extensive bioassays have shown that both NNN and NNK are potent carcinogens and induce organ specific cancers in mice rats and hamsters. The organ specificity is directed to the nasal cavity, trachea, esofagus, and liver through different routes of administration, but NNN and NNK are also potent local carcinogens in the oral cavity (16, 45).

It has been reported, however, that after swabbing of the oral cavity with NNN and NNK significantly more tumors were induced in the oral cavity and the lungs, than after swabbing with a snuffextract containing the same concentrations of NNN and NNK. This indicates the presence of inhibitory agents to the tumorigenic activity of the TSNA in snuff (16, 45).

Chemical Carcinogenesis

Attempts to chemically induce malignant tumors of the oral squamous cell epithelium in rats were fruitless, until Fujino in 1965 introduced the water soluble carcinogen 4-nitroquinoline N-oxide (39). An experimental model of oral cancer was described by Wallenius and Lekholm in 1973. They produced squamous cell carcinomas of the palatal mucosa in rats by repeated applications of 4-NQO. The carcinogenicity potential of 4-NQO is now well documented, and it has been shown to induce oral and squamous cell carcinomas as well as spindle cell sarcomas of various rodent species (36, 118, 125).

There is considerable evidence that carcinogenesis proceeds as a step-wise series of cellular changes which leads to the development of neoplasia. This idea was first proposed by Rous and Kidd, who observed that papillomas of rabbit skin induced by repeated applications of tar would regress on the cessation of these application, but could be caused to reappear by the application of an irritant such as turpentine (95). The multiple steps in the developmen of cancer can be divided into two major phases, initiation and promotion. The discovery of a two-stage mechanism of carcinogenesis in mouse skin with initiation and promotion as independent components has provided

new approaches to the study of the development of neoplasms (34, 58). It has also been shown that papillomas induced by repeated treatments with initiating doses of carcinogens arise from significantly more cells than those induced by a carcinogen promoter regimen (1983).

Steidler and Reade reported 1986 that 4-NQO induced epithelial dysplasias after as little as 2 weeks and oral squamous cell carcinomas in all mice treated for 12 weeks with 4-NQO followed by application of the tumor promoter phorbol-12,13-didecanoate (TPA) (109).

Wynder et al 1957 applied the terms intrinsic and extrinsic to group factors which act together to produce a malignant transformation. Extrinsic factors are exogenous and have local effects such as tobacco and sunlight. Intrinsic factors infer generalized defects from such things as malnutrition from alcoholism, vitamin deficiencies and sideropenia (9, 127). It is a well established fact that alcohol consumption increases the risk of epithelial cancer of the oral cavity, oropharynx, larynx and esophagus among tobacco smokers (19, 70, 113, 114).

Herpes Simplex Virus Type 1

HSV-1 belongs to the herpes virus family which consists of more than 50 herpes viruses, capable of infecting more than 30 different species. The genome of HSV-1 consists of linear double stranded DNA, 152 260 base pairs with an approximate weight of 100 million. The G+C content is 68 per cent. The genome is built up of two components, long and short, which are covalently linked. Both the uniqe short segment, U_S, and the unique long segment, U_l, are surrounded by inverted repetitive sequences enabling the DNA to circularize (94, 117).

The expression of the HSV genome in infected cells is sequentially regulated in cascades of protein synthesis. The five groups of proteins are designated α-,ß1-, ß2, τ1-, and τ2-proteins - of which α- and ß-proteins are synthesized prior to progeny HSV DNA (55, 94). α-protein synthesis requires no prior protein synthesis but ß-proteins require previous synthesis of α-proteins, and τ-proteins require previous ß-protein synthesis for their production.

A significant property of all herpes simplex viruses is their ability to establish a latent infection, i. e. persist in an inactive state for a varying duration of time in the infected host and then reactivate when provoked by a proper stimulating agent. The mechanisms for establishment of latency as well as the reactivation and maintenance of the latent state are still unknown (38, 93).

Herpes Simplex Virus; Epidemiology

Primary HSV-1 infections are mostly asymptomatic and occur predominantly in young children. Gingivostomatitis is the most common clinical manifestation. Primary infection in young adults has been associated with only pharyngitis but is mostly associated with a syndrome resembling mononucleosis. Herpes simplex viruses have a worldwide distribution and have been reported in both developed and developing countries. Humans are the only sources of infections and the virus is transmitted from infected to susceptible individuals during close personal contacts. Due to the widespread existence of HSV and the frequency of asymptomatic disease are epidemiological studies difficult to perform when based on clinical history only (121). A major problem in the serological investigations of HSV prevalence is the cross reactivity between HSV-1 and HSV-2. The utilization of type specific antigens (gG-1 and gG-2) in serological studies has enabled more distinguished epidemiological studies on the prevalence of HSV-2 (64, 111).

HSV-1 is a ubiquitous virus and a great majority of the adult population in North America and Europe are seropositive and carriers of latent infections. However, geographic location, socioeconomic status, and age considerably affect the prevalence of HSV antibodies. In lower socioeconomic groups, 90 per cent of the population are carriers of latent infection (75-77). In Brazilian Indians 95 per cent of children aged 15 had antibodies against HSV, and in certain poor urban areas in the United States similar frequencies of seroprevalence of HSV antibodies among children have been obtained (8). Middle class individuals acquire antibodies later in life. Seroconversion occurs in 20 per cent of the children during the first five years of life, followed by no significant increase until the second or third decade of life when prevalence of antibodies increased to 40 and 60 per cent, respectively (119). In Gothenburg, Sweden, there is a 65 per cent prevalence of antibodies against HSV-1 in blood donors, with an average age of 30 years and of which 70 per cent are males (T. Bergström , personal communication).

Reactivation of herpes simplex virus (HSV) in the trigeminal ganglia and peripheral shedding of virus in the mouth is frequent and not necessarily accompanied by epithelial lesions (26). Recurrent lesions are experienced by approximately 40 per cent of the infected population (103) and these lesions occur despite the presence of circulating HSV neutralizing antibodies (26). New lesions occur in a frequency of one per month in five per cent of the population and with intervals of two to eleven month in 34 per cent of the population (121).

Herpes Simplex Virus; Cell Transformation and Cancer

Reaserch on cancer etiology was early directed towards virus as possible initiators of neoplasia. As early as 1908 Elleman and Bang in Denmark had been able to transfer erytromyeloblastosis to chicken with cell-free suspensions and Peyton Rous of the Rockefeller Institution reported in 1911 that cell-free filtrates from chicken tumors called sarcomas could induce new sarcomas in chicken. A causal association between a herpes virus and renal adenocarcinoma of the leopard frog was suggested by Lucké in 1938 (67). Decades later a herpes virus was identified as the causative agent of Marek's disease, a lymphoproliferative disease of chickens with important economic consequences for chicken production (20). As these and a some other animal cancers are caused by herpes viruses under natural conditions it could be that also humans might be affected by herpes viruses in a similar way (85).

In particular the Epstein-Barr virus (EBV) has been thoroughly investigated as the causative agent of Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC) (33). EBV was first isolated from a child with BL (32) and has the capacity to transform human B-lymphocytes to continously growing cell lines (47). BL patients have higher levels of antibodies against a wide range of EBV-determined antigens, than do matched controls (46). Extensive epidemiologic investigations in Uganda revealed that children who developed BL during the investigation had earlier been shown to have higher antibody titers to EBV capsid antigens (24). EBV nuclear antigen and EBV DNA have been detected in BL-tissue (85). EBV DNA has also been found in an integrated state in EBV transformed human cell lines (3). EBV DNA and EBV nuclear antigens have also been identified in tissue specimens from nasopharyngeal carcinomas and it has been suggested that expression of the EBV genome is regulated in a tissue specific fashion (40). However, in view of the restricted geographical distribution of BL and NPC and the ubiquity of EBV, other co-factors must also be considered in the etiology of BL and NPC. It has been reported that

some food, in highly endemic areas of NPC (harissa, a spice mixture from Northern Africa and Cantonese salted dry fish), have the capacity of activating EBV in Raji cells, which bear latent EBV genome (99).

Compared to EBV, analogous evidence of the association between HSV and human cancer is less complete. Study of the association between HSV and human cancer is made difficult by the widespread existence of the virus in the general population.

Two human cancers have been associated with HSV, oral cancer and cancer of the uterine cervix. The earliest reports of an association between HSV and human cancer were published by Wyburn-Mason (126) and Kvasnicka (62), who reported a total of eight patients in whom carcinoma of the lip had developed at the site of a previous frequent herpetic infection. Their reports were entirely anecdotal, and the cases were selected from a patient population of unknown size. No confirmatory reports have been published since. Lehner et al. have reported that patients with oral leukoplakias exhibiting dysplasia had an increased cell-mediated immune response to HSV-1 compared to patients having leukoplakia without dysplasia (65, 66). Shillitoe and Silverman stated in 1979 that there was no clinical association between recurrent herpes labialis and cancer of the lip or mouth, but since HSV can be carcinogenic or co-carcinogenic in laboratory experiments under certain circumstances, it must be considered as a possible etiologic agent in oral cancer (100). However, fragmentary evidence has accumulated to suggest a connection between HSV-1 and cancer of the mouth.

An association between HSV-2 and cancer of the uterine cervix has also been proposed, based on several epidemiological studies reporting significantly increased frequency of antibodies against HSV-2 among women with preinvasive or invasive cervical cancer (72, 74, 86, 87). However, attempts to isolate a transforming gene from HSV 2 have however given inconsistent results (68). The results of more recent epidemiological studies have also suggested that infection with HSV-2 is a co-variable of venereal risk factors although a role for the virus in the genesis of a certain proportion of cervical cancers can not be excluded (88). HSV-2 antigens have been detected in cervical cancer cells (5, 6, 112).

Oral cancer is associated with raised levels of IgA and IgM antibodies to some proteins of HSV-1 (106, 101, 102) and RNA of HSV-1 has been demonstrated in some oral tumors (30). Furthermore, HSVá1 specific proteins have also been demonstrated in oral cancer tissue by immuneperoxidase technique (57, 61).

The oncogenic capacity of HSV is well documented in experimental systems. Both HSV-1 and HSV-2 can transform cells in vitro (23, 28, 29, 41). Syngenic animals inoculated with the transformed cells may acquire malignant tumors. However, a prerequisite for HSV to cause cell transformation is that the virus induced cell-lysis is prevented (85). In an initiation-promotion study a mouse model for lip carcinogenesis was used to combine HSV-2 infection, ultraviolet irradiation and applications of a tumor promoter (17). It was found that ultraviolet irradiation of the HSV lesion site on day 3, 4, 5, and 6 post infection caused hyperkeratosis, acanthosis, and dysplasia in several lips. The addition of repeated TPA applications to the HSV inoculated and ultraviolet irradiated lips resulted in tumor development. Burns and Murray concluded that the inactivation of HSV by u.v. irradiation after infection allowed the virus to express its inherent oncogenic capacity when combined with a tumor promoter.

A co-carcinogenic effect of HSV in vivo has been shown by Southam et al (108). When the skin of mice was painted with a low concentration of the hydrocarbon 3-methylcholanthrene, a low incidence of skin tumors was found. The simultaneous application of HSV significantly increased the yield of tumors and also increased the proportion of tumors which became malignant.

Herpes Simplex Virus and Tobacco

The interaction of tobacco extracts with HSV-1 replication has earlier been studied. Aqueos extract of snuff has been shown to dose dependently inhibit the replication of HSV-1 in in vitro cultured cells. The block in HSV replication induced by snuff extract is an early function - i.e. before or at the level of DNA replication (50, 52, 110). Similar results have also been obtained when HSV infected cells were exposed to water extracts of condensated smoked tobacco tar (80). Since the prevention of virus-induced cell lysis is a prerequisite for HSV to cause cell transformation, substances, which inhibit HSV replication and which are held in the mouth for prolonged periods of time, may be of potential danger for the development of malignancies.

In animal models, the life-long effects of simulated snuff dipping and acute infections with HSV-1 and HSV-2 have been assessed, and rats and hamsters exposed to the joint action of tobacco and virus developed malignant tumors (51, 81). An Australian epidemiological study of smoking and infectious agents as risk factors of in situ cervical carcinomas showed that smoking was the major risk factor, but also that the

patients appeared to have more exposure to HSV-1 as measured by antibody prevalence. Exposure to HSV-2 and CMV was unrelated to risk (14).

An interaction between tobacco products and HSV-1 infection in the development of cancer in the head and neck region has been suggested (51), as has HSV-2 infection and smoking for cancer of the uterine cervix (122).

METHODS AND METHODOLOGICAL CONSIDERATIONS

Animals and Animal Model (I-IV)

Three-month-old male and female Sprague Dawley rats were used in study I-III. Inbred Lewis rats were used in two of the six groups of the study reported in paper III. Seven-week-old, Swiss-albino mice, own bred were used in study IV. The animals were kept in plastic cages, male and female separately. The animals were fed a standard pelleted diet and tap water ad libitum. Temperature in the animals' quarter was kept constantly between 21 and 23° C, the relative humidity was 50 ± 20 per cent and the light/dark cycle was 12 hours constantly. The model for snuff exposure in rats (I-III) has earlier been described in detail (48). The administration of 4-NQO, TPA and acyclovir is described in the separate papers.

The average body weight of the three month old rats at the beginning of the experiments was approximately 375 grams. The average weight in all groups increased during the first one-and-a half years of the experiments but snuff treated groups had a significantly slower weight gain than groups not receiving snuff. The weight of snuff treated rats was after 40 weeks 100 grams lower than of not snuff treated groups, and this difference remained until the end of the experiments. It was also observed that snuff treated animals consumed less food than not snuff treated, but this difference was not statistically significant. Water consumption was not affected by snuff treatment but increased with age in all groups, which may be related to the pronounced rat nephrosis present in the majority of the rats at the time of sacrifice. Rat nephrosis is a major factor underlying health detoriation and death in ageing laboratory rats, especially male Sprague-Dawley. (42). Lewis rats survived six months longer than Sprague-Dawley rats which may be explained by a much less pronounced rat nephrosis in these rats.

The latent period for tumor development in HSV infected mice treated with TPA and acyclovir was 11-14 months. Consequently, one problem was decreasing general health with a concomitant loss of mice. However, we considered the long term treatment and follow up period important and therefore accepted the high mortality among the animals.

Morphological Methods(I-IV)

Histological examination was performed on the lip, gingival epithelium of the lower incissors (crevicular epithelium), tongue and buccal mucosa in study I while all animals in studies II, III, and IV underwent complete autopsy for the recording of tumors and other pathological lesions. Specimens from the lips, test canal, palate, oral and nasal cavities, lungs, heart, liver,esophagus, forestomach, glandular stomach, kidneys, urinary bladder, and other grossly abnormal tissues were taken for light microscopic examination. Tissue specimens were fixed in 4 per cent neutral, buffered formalin solution, embedded in paraffin, sectioned and stained by routine methods, hemaoxylin-eosin and according to Weigert van Gieson. Immunochemical staining with antibodies against keratin (MAK-6) and vimentin was performed on selected cases (II).

Cells Viruses and Chemical Substances

Green monkey kidney (GMK) cells and human epidermoid carcinoma No.2 (HEp-2) cells, were used in the experiments. The HSV-1 strain F was used in the protein synthesis assays and the MacIntyre strain was used in the viral replication assays. The technique for preparation of virus stock suspensions and for the plaquing of virus in GMK cells have earlier been described in detail (116) Water extract of snuff was prepared as described earlier (52) from fresh Swedish snuff purchased on the open market in Gothenburg. The aqueous extract of snuff was analyzed for the content of the tobacco alkaloids, nicotine, anabasine, and anatabine and the tobacco specific nitroseamines NNN and NNK. - NNN and NNK was a kind gift from Dr. D. Hoffmann, American Health Foundation, New York. All other chemical and radiochemical substances were purchased from diffrerent commercial sources.

Cell-toxicity of tobacco alkaloids, DEN, NNN, and NNK was assessed by three different methods: a; Daily light microscopic monitoring of morphological appearance of cells, b; Cell counting performed on a coulter counter to study the growth rate of cells, and c; Analysis of cellular protein synthesis by a dye-binding assay earlier described by Bradford (12).

Assay of HSV Attachment to Cells (V)

GMK cells were grown as confluent monolayers in a 96 well microtiter-plate and allowed to adsorb HSV at an MOI of 500 PFU /cell. After intervals ranging from 0 to 120 min virus suspensions were discarded and cells washed five times with 0.15 M phosphate buffered NaCl (PBS) and fixed in 0.02 percent formaldehyde. Adsorbtion of virus was determined with an ELISA, using a monoclonal mouse antibody against HSV-1, alkaline phoshpatase conjugated goat anti-mouse IgG. After the addition of substrate, adsorbtion was read in a Biotek microplate reader at wavelegths 405 and 540 nm. Adsorbtion curves were plotted versus the time of attachment (52). The inhibitory effect of snuff extract on HSV attachment to cells could not be attributed to any of the tobacco chemicals studied. However, in all experiments on HSV replication and virus protein synthesis, snuff extract or tobacco chemicals were added one hour post infection in order not to interfere with the HSV adsorption to the cells.

HSV Production Assay (V)

GMK cells were inoculated with 0.5 ml of virus suspension at an MOI of one PFU per cell, or as otherwise stated. In order not to interfere with the attachment of virus to cells, virus was allowed to adsorb to the cells for one hour at room temperature before tobacco chemicals and snuff extracts were added. The cells were then washed three times with Eagle's MEM, incubated at 37°C for 24 hours with added test substances dissolved in three ml of maintenance medium. The dishes were frozen and thawn rapidly in three consecutive cycles after which cells and medium were transferred to a centrifuge tube and centrifalized for 10 minutes at 1000 rpm to remove cell debris, whereafter plaque titration was performed to measure the progeny virus production. 500 μl of supernatant diluted from 10^{-1} to 10^{-6} was seeded on GMK cultures in duplicate. After 30 minutes the cultures were covered with plaquing medium containing 1 per cent methylcellulose. Plaques were counted after five days.

Assay of Protein Synthesis (V)

Confluent HEp-2 cells in 5 cm Petri dishes were inoculated with HSV at an MOI of 20 PFU per cell. After one hour of incubation at room temperature cultures were rinsed three times with Eagle's MEM and exposed to snuff extracts and chemicals at the concentrations stated above. For the labelling of immediate early proteins (α-

proteins), cultures were incubated with [^{35}S]-methionine, approximately 25 μCi/culture, dissolved in methionine free Iscoves medium, (3ml/culture) containing snuff extracts or tobacco chemicals, from one to four hours post infection. To study the production of early proteins (ß-proteins), the infected cells were incubated for three hours in 5 per cent CO_2 at 37°C in maintenence medium with snuff extracts and tobacco chemical added as stated above. After this the cultures were rinsed and labelling was performed in the same way as for α-proteins from four to eight hours post infection. For labelling late proteins (τ-proteins), infected cultures were incubated for seven hours in 5% CO_2 at 37°C in maintenence medium with snuff extract and tobacco chemicals added and labeled from eight to twelve hours post infection as described above. At the end of the labeling period the cultures were rinsed three times with ice cold PBS, to terminate amino acid incorporation, harvested with a rubber policeman, dissolved in a small volume of PBS and centrifugalized for four min at 3000 rpm 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 per cent sodium dodecyl sulfate (SDS), 5'ß-mercaptoethanol, and 0.05 M Tris-hydroxychloride (pH 7.0). Fifty μl of the solubulized material from each culture was added to each well of the gel. In parallel, 50 μl of each sample was precipitated onto filter papers with 10% ice cold TCA. The precipitate was washed twice with 6% TCA, once with with ethanol/ether mixed in proportions 50:50 and once with ether only. The radioactivity of the TCA precipitates on dried filter papers was assessed by liquid scintillation.

Polyacrylamide gel electrophoresis was performed as described by Morse et al. (73), in a discontinous buffer system containing 0.1 per cent SDS. The stacking and separation gel contained 3 and 9 per cent acrylamide respectively, cross linked with N,N'-diallyltartardiamide (2.6 per cent of acrylamide weight). All chemicals for gel preparation were purchased from Bio-Rad, Richmond, Ca., USA. Separation gel was 15 cm in length. Proteins used for molecular weight calibration were 14C-methylated-myosin, phosphorylase-b, bovine serum albumine, ovalbumin, carbonic anhydrase, and lysozyme (Amersham International, Amersham, England) 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 mw 100 000 and 92 500. Protein bands were designated according to Morse (73). Adsorbance measurements of the autoradiographic images were performed in a Shimadzu CS 910 spectrophotometer equipped with a CR 1B chromatoscan. Amount of each protein was recorded and calculated as per cent of total adsorbance in lane, as well as per cent of TCA precipitable radioactivity added to the lane. The overall relationship of

each individual protein between the variously treated cultures did not differ, no matter which of these methods was used.

To assess the production of viral thymidine kinase and DNA-polymerase in infected cells a bioassay was used measuring virus specific enzyme activity which indirectly reflects produced amounts of these enzymes. Cultures were infected with HSV yielding an MOI of 1 PFU/cell. Virus was adsorbed for one hour at room temperature after which cells were rinsed three times and incubated in 5 per cent CO_2 at 37°C in maintenence medium with added snuff extracts and tobacco chemicals. After one, four, seven and eleven hours respectively, duplicate cultures were rinsed three times with ice-cold PBS, scraped off with a rubber policeman, dissolved in one ml of PBS and frozen at -70°C. Enzyme assays for the determination of DNA polymerase and thymidine kinase activity were performed as described earlier (43, 78).

Patients (VI)

The material of study VI consisted of 90 patients with carcinomas of the head and neck region and 79 control patients. The non tumor bearing control patients were selected among patients treated for ischemic manifestations in their lower limbs of arteriosclerosis. Cancer of the head and neck region is strongly associated with extensive smoking habits, and mainly affecting the elderly people. The rationale when chosing the control group was to find a group of patients of matching age and sex distribution as well as smoking habits. The mean age in the material was 64.1 ± 1.04 years and the sex distribution was 74.0 percent men and 26.0 per cent women. The proportions of men and women did not differ significantly in the groups of cancers and controls, with the exception of the group with larynx cancer, in which the proportion of women was significantly lower. All tumours were investigated histopathologically. The patients with carcinomas were divided into three groups according to the anatomic location of their tumors, patients with laryngeal cancer, (n=16), patients with oral cancer (n=28), and patients with cancers of other types and locations in the head and neck region (n=46). The tobacco habits of each patient were elicited by means of questionnaires. These revealed that 78 per cent of the cancer patients were or had earlier been tobacco users. In the control group 89.9 per cent were or had earlier been tobacco users. Three patients with different types of head and neck cancer and one control patient were snuff dippers, one patient with laryngeal cancer and four control patients were both snuff dippers and smokers. All other cancer patients and controls were cigarette smokers. The most extensive

tobacco habits were found in the control group while the group with unspecified head and neck cancers had the highest proportion of non tobacco users. In the group of laryngeal cancer patients all had a history of tobacco consumption.

Serological Methods (V)

Sera were collected from all cancer patients as soon as the cancer diagnosis was certain and before treatment was begun. IgG titers against HSV, HSV-2, CMV, and VZV were established by an ELISA based technique described previously (111). Titers were expressed as the reciprocal of the dilution giving an adsorbance higher than three SD above mean of negative controls. The tests were performed on a 96 well plastic dish. One positive control, two negative controls, and ten test samples were analyzed on each plate and all samples were analyzed parallelly on two different occasions. Titers less than 100 were regarded negative. The antigen used for the detection of antibodies against HSV was a sodium deoxycholate solubilized antigen prepared from HSV-1 infected cells which is not type specific, but the antigen used for detection of HSV-2 antibodies is a lectin purified type specific antigen. Sera from patients with verified HSV-2 infection crossreact in approximately 75 per cent of the cases with the HSV-1 antigen. The antigens used for the assays of antibodies against VZV and CMV were prepared as described earlier (37, 60).

Immunoblotting was performed according to Braun et al.(13). Two ml of [^{35}S]-methionin-labeled HSV-1 infected Hep-2 cell extract was separated in 9.25% polyacrylamidgels (73). The proteins were transferred to nitrocellulose papers (pore size 0.45 μm) by means of a Bio-Rad transblot cell (13). The transfer was cut in 0.5 cm wide strips. The strips were placed in a Bio-Rad small incubation tray. Each strip was washed three times in PBS containing 0.05% Tween 20 and treated for 30 minutes with PBS supplemented with 3 per cent BSA, 4 per cent FCS and 0.05 per cent Tween 20. Each serum was dispersed in this medium to a dilution of 1:100. Incubation time was 18 hours followed by three washes in PBS with 0.05% Tween 20. Thereafter strips were incubated with horseradish peroxidase-coupled rabbit anti-human IgG (DAKO, Copenhagen, catalogue No. P214) for 2 hours using a 1:200 dilution of the conjugate in PBS with 3% BSA 4% FCS and 0.05% Tween 20. The antibody binding was visualized after washing and addition of 4-chloro-1-naphtol (Bio-Rad, Richmond, CA., USA). Sera not reacting against ICP 4 at dilution 1:100 were retested at dilution 1:20.

Statistical Methods.

The data are presented as mean ± SE. The statistical significance of differences between group means, unpaired samples, were calculated with the Student's T-test and the significance of differences in proportions between groups was calculated with the Fischer's exact test and the chi square test. A p-value less than 0.05 was regarded statistically significant. The tumor incidence in the animal studies (paper II-IV) was calculated in each experimental group and factor analysis was made for the chemical compounds studied, as well as for the combinations of these (21).

RESULTS

Reversibility of the Snuff Induced Lesion in Rat Oral Mucosa (I)

The squamous epithelium of the lips of the test group killed immediately after thirteen months snuff exposure exhibited a generalized slight (40 per cent) or moderate (60 per cent) hyperplasia. Hyperorthokeratosis was observed in all animals and in certain parts the hyperorthokeratosis was marked while in others a looser type was seen with focally vacuolated cells extending down into the stratum granulosum (50 per cent). Slightly (80 per cent) or moderately (20 per cent) acanthotic proliferations with the development of marked rete pegs were noted. The squamous epithelium showed mild focal atypia (40 per cent) as well as focal ulcerations (20 per cent), but the border between the stratum basale and the connective tissue was always well defined. The inflammatory reaction (mostly lymphocytic infiltrates) in the underlying connective tissue was slight (60 per cent) or severe (40 per cent), but above all a prominent fibrosis was noted (100 per cent). In the two test-groups, killed after a snuff free intervals of one and four months, respectively, histopathological changes in the test canals were less prominent. Thus, in comparison with the test group, killed immediately after cessation of snuff exposure, only one rat in each of these two groups exhibited ulcerations. The lesions were more atrophic after a snuff free interval of one month and four months, with slight or no acanthosis. The inflammatory reaction was slight or absent in both these groups. Mild atypia of squamous epithelium was only seen in one rat in the group killed four months after the cessation of snuff treatment. In 60 per cent of the rats killed after one month, and in all the rats killed four months after the cessation of snuff treatment, severe subepithelial fibrosis was observed.

Moderate or severe hyperplasia with increased keratinization was observed in the epithelium, lining the gingival sulcus, in rats exposed to snuff for thirteen months and then killed immediately or after one month. Focal resorption of the marginal bone plate buccally to the lower incissors, was also noted in one case in each of the groups killed immediately or one month after cessation of treatment . Apart from these findings, the majority of the specimens exhibited atrophy and focal ulcerations (70 per cent) of the gingival sulcus epithelium. The rats killed four months after termination of the snuff exposure, exhibited only slightly hyperplastic epithelium of the gingival sulcus (70 per cent) with little or no keratinization. The epithelial atrophy was less,(30 per cent), and only occasionally, ulcerations were seen. The light microscopic

appearance of the squamous epithelium of the gingival sulcus of the control animals, not exposed to snuff, did not show any noteworthy pathological changes.

Tumour Incidence in the Lips and in the Oral Cavity in Rats Exposed to Snuff, 4-NQO and HSV-1 (II, III)

In study II, two carcinomas of the oral cavity, one lip carcinoma, and two lip sarcomas were found in the group of rats exposed to snuff only (n=29). In the group exposed to 4-NQO only (n=29), four carcinomas of the oral cavity were registered and in the group exposed to the combination of 4-NQO and snuff (n=28) five carcinomas of the oral cavity and three sarcomas of the lip were observed. In the control groups exposed only to propylenglycol (n=28) or cotton pellet (n=29), no tumors of the lips or oral cavity were found. In study III, one lip cancer was found in the group exposed to HSV only (n=12), one intraoral carcinoma was found in the group exposed to snuff only (n=13) and one salivary gland carcinoma was found in the group exposed to the combination of HSV and snuff (n=15).

General Tumor Incidence in Rats Exposed to Snuff, 4-NQO and HSV-1 (II, III)

In study II, the overall tumor incidence was highest in the groups exposed to snuff, with 23 tumors being presented in the group exposed to snuff only (n=29) and 22 tumors in the group exposed to the combination of snuff and 4-NQO (n=28). In the group of rats exposed to 4-NQO only (n=29), 13 tumors were found. In the control group exposed to propyleneglycol only (n=28), five tumors were found and in the control group, only undergoing the surgical procedure (n=29), three tumors were found.

In study III, one leukemia was found in the control group of eight rats but no solid tumors. In the group exposed to snuff only (n=13), three tumors were observed and in the group exposed to HSV only (n=12) four tumors were registered. In the group of rats exposed to the combination of HSV and snuff (n=15) thirteen tumors were found.

Tumor incidence in HSV-1 infected mice, treated with acyclovir and 12-O-tetradecanoylphorbol 13-acetate (IV)

Twentyfive per cent of the mice exposed to the combination of HSV infection and TPA application, as well as 25 per cent of the mice exposed to HSV TPA and acyclovir treatment developed tumors. Twelve per cent of the control animals developed tumors. None of the animals exposed to the combination of acyclovir and TPA treatment developed tumors, and only 4 per cent of the animals exposed to HSV and acyclovir treatment without the addition of tumor promoter developed tumors. Thus, the combination of HSV-1 infection and TPA treatment induced a significant increase of tumor development, but the inhibition of the simulated recurrent HSV infection with acyclovir did not inrease the incidence of tumor development in mice. In this study the tumors developed after 11 to 14 months. TPA administration was discontinued after 13 months, two months before discontinuation of the study, which may have resulted in the disappearance of reversible lesions induced by TPA.

Effect of Tobacco Extracts, Nicotine, Anabasine, DEN, NNN, and NNK on GMK cells (V)

Confluent GMK-cells cultures were exposed to snuff extract and snuff extract diluted 1:5 and 1:25 for six days. Morfological signs of toxicity could be detected after 4 days in cultures exposed to undiluted snuff extract and after six days slight signs of toxicity were seen also in cultures exposed to snuff extract ta a dilution 1:5. No morfological signs of toxicity were detected in cells exposed to snuff extract in dilution 1:25. Clear morfological signs of toxicity were detected after three days in GMK cultures exposed to 1.0 mg/ml of nicotine, anabasine, and DEN. Slight toxic effects were also seen in cultures exposed to 1.0 mg/ml of NNN and NNK after six days. No morfological signs of toxicity could be detected in cultures exposed to 0.1 mg/ml of the drugs used in these experiments.

Snuff extract at dilution 1:5 exerted a 33 per cent decrease in cell growth and a 50 per cent decrease in cellular protein production while snuff extract diluted 1:25 stimulated cellular division yielding a 31 per cent increase in cell number, despite a slight reduction of protein synthesis. Growth rate of GMK cells was affected by undiluted snuff extract and nicotine, anabasine NNN, NNK, and DEN at concentration 1 mg/ml The slight depressive effect on cell growth implied by nicotin, anabasine and NNN at concentration 0.1 mg/ml was not statistically significant. Neither was the slight

stimulating effect on cell growth induced by NNK in concentration 0.1 mg/ml statistically significant.

Effect of Snuff Extract, Nicotine, Anabasine, DEN, NNN, and NNK on the Attachment of HSV to Cells (V)

The kinetics of the attachment of HSV to cellular receptors in the presence of snuff extracts, nicotine, anabasine, DEN, NNN, and NNK were studied. Snuff extract diluted 1:2 induced a complete inhibition of attachment and also at dilution 1:5 inhibition of attachment was significant. Nicotine induced a slight reduction of HSV adsorbtion to cells. Neither NNN, NNK, DEN nor anabasine had any effect on the attachment of HSV to cellular receptors in any of the concentrations tested.

Effect of Tobacco Extracts, Nicotine, Anabasine, DEN, NNN, and NNK on the HSV Replication in GMK cells (V)

In GMK cultures inoculated with HSV-1 at an MOI of 1 PFU/cell snuff extract at the dilution 1:5, production of progeny virus was reduced by three log units whereas snuff extract at the dilution 1:25 implied a one log unit reduction of progeny virus (more than 90 per cent) compared to the controls. The reductions induced by snuff extract at dilution 1:5 and 1:25 are both statistically significant. GMK cultures were also inoculated with HSV-1 at different MOIs and after a one-hour-interval of virus adsorbtion exposed to snuff extract in dilution 1:10. The results of this assay indicated that the depressive effect on HSV production induced by snuff was to some extent dependent on the multiplicity of infection. Nicotine at a concentration of 1.0 and 0.5 mg/ml significantly reduced the production of HSV but not at lower concentrations. Also anabasine at the concentration 1.0 mg/ml, and DEN at the concentration 1.0 mg/ml induced a significant reduction of HSV production, but, the effects of anabasine and DEN at lower concentrations on HSV progeny production were not statistically significant. Neither were the effects of NNN and NNK.

Effect of Tobacco Extracts, Nicotine, Anabasine, DEN, NNN, and NNK on HSV Protein Synthesis (V)

Snuff extract, both at dilution 1:5 and 1:25 induced an increase in the production of immediate early proteins ICP 4 and ICP 27 and also the early proteins ICP 6 and ICP 8 measured up to eight hours post infection. The production of late proteins was reduced by the addition of snuff extracts at the dilution 1:5 to HSV infected cells, measured eight to twelve hours post infection. This reduction remained until 24 hours post infection. At the dilution 1:25 the effects on the production of late proteins was a slight reduction of ICP 5, ICP 11, and ICP 21, but in the production of ICP 29, there was a slight increase . Although nicotine at the concentration 0.5 mg/ml enhanced the production of ICP 4, ICP 27, and ICP 6 measured one to four hours post infection, it had no detectable effect on late protein synthesis. Anabasine, DEN, NNN, and NNK had no significant effect on HSV α- and τ-protein synthesis when given in non-toxic doses.

Activities of the HSV induced DNA polymerase and thymidine kinase were studied with bioassays as indicators of the production of these two ß-proteins. The activity of the DNA polymerase and thymidine kinase increased from one to eleven hours post infection. Eleven hours post infection the activity of DNA-polymerase was reduced 97 per cent by snuff extract at the dilution 1:5 and 77 per cent by snuff extract at dilution 1:25. The reduction in thymidine kinase activity eleven hours post infection was 88 per cent when exposed to snuff extract at dilution 1:5 and 72 per cent when exposed to snuff extract at dilution 1:25. Also nicotine and anabasine at a concentration of 1.0 mg/ml exerted a depressive effect on the activity of thymidinkinase and DNA polymerase, but had no significant effect when tested at lower concentrations. Neither DEN, NNN nor NNK had any effect on DNA-polymerase and thymidine kinase activities.

Effect of Tobacco Extracts, Nicotine, Anabasine, DEN, NNN, and NNK on Synthesis of the Cellular Protein Actin (V)

The autoradiographic images of SDS-PAGE revealed that the decrease in the production of the cellular protein actin four hours post infection and later, in HSV infected cells, was significantly affected by the addition of snuff extracts to the culturing medium. From four to eight hours post infection, the relative synthesis of actin was 55 per cent higher in cultures exposed to snuff extract at a dilution of 1:5

than that in control cultures. Also in cultures exposed to snuff extracts at the dilution 1:25, the actin production from four to eight hours was slightly but not significantly higher than in control cultures. From eight to twelve hours post infection the production of actin still contributed to 7.5 per cent of total protein synthesis in cultures exposed to snuff extract in dilution 1:5 and 2.2 per cent of total protein synthesis in cultures exposed to snuff extracts at dilution 1:25. No significant effect on the actin production in HSV infected cultures was observed when non-toxic doses of nicotine, anabasine, DEN, NNN, or NNK were added.

Antibody-Prevalence and IgG Titers against HSV, HSV-2, CMV, and VZV (VI)

The prevalence of IgG-antibodies against HSV, HSV-2, CMV and VZV did not differ significantly between the groups, but the prevalence of anti HSV IgG positive patients was unexpectedly high (more than 90 %). Antibody levels against VZV did not differ significantly between the groups, whereas antibody titer against CMV was significantly higher in the control group compared to the groups of cancer patients. Antibody titers against HSV-1 was signficantly higher in all groups of cancer patients compared to the control group, with the highest titers found in the groups of oral and laryngeal cancers, which were predominantly squamous cell carcinomas. The average anti HSV IgG log titer was 4.19 ± 0.05 in the cancer patients and 3.84 ± 0.06 in the control group.

Immunoblotting (VI)

The immunoblotting of sera confirmed the results of prevalence of antibodies against HSV-1 obtained by ELISA. Two sera, one from a patient with laryngeal cancer, and one from a control patient, which were negative in ELISA, reacted positively in Western blot. Most sera bound strongly to several HSV-proteins, but variability among individuals was observed. Sera from cancer patients reacted more constantly to the different HSV proteins, especially against the early immediate protein ICP 4, compared to sera from control patients. More than 80 per cent of sera from cancer patients reacted to ICP 4, compared to 53 per cent of control sera. Reactivity to ICP 4 did not differ between smokers and non-smokers or between male and female patients.

GENERAL DISCUSSION

Non smoked tobacco is carcinogenic, but as shown here and in several earlier studies, the carcinogenic effects are weak (16, 45, 54, 56, 124, II). Furthermore, the lesions induced by snuff in rat oral mucosa are to a certain extent reversible after 13 months of snuff exposure (I). The effects of snuff on the oral mucosa with hyperplasia, hyperorthokeratosis, atypia, and fibrosis suggests a stimulating effect on reactive cellular proliferation which could be related to tumor promotion.

Whether the major effect of snuff is initiating or promoting is not clear. Although there was a slightly higher incidence of tumors in rats exposed to a low dose of 4-NQO in the combination with snuff compared to 4-NQO only (II), this was not statistically significant. Furthermore, the number of tumors were almost identical in the groups exposed to snuff only and the combination of 4-NQO and snuff (II). Thus, it seems unlikely that snuff acts only as a tumor promoter although it does not rule out such an activity.

One factor which may interact with non smoked tobacco in carcinogenesis is HSV infection (50, 52, 81, III). It is not known which of these agents is initiating or promoting. However, it has been clearly demonstrated that the combination of HSV infection and tobacco has a carcinogenic effect (81, III). The general tumor incidence in rats exposed to the combination of HSV infection and snuff (III) was markedly increased. However, a localized tumorigenic effect of HSV at the site of application as earlier reported (17, 51) was not observed in the present investigation (II). The reasons for this is uncertain, but substances present in snuff might act as local inhibitors of the cancer development. Polyphenols have been ascribed such an inhibitory activity (10, 16, 45). Another explanation is that the multiplicity of infection was too high, resulting in cell lysis in the oral mucosa (V).

Among several possible mechanisms to account for the higher tumor incidence in rats exposed to the combination of HSV and tobacco is that the inhibition of the HSV replication and subsequent cell lysis by snuff (52, 110) enabled HSV-1 to express its possible carcinogenic properties. This hypothesis is supported by several earlier reports showing that HSV, when inactivated by ultraviolet light or neutral red and light, can transform cells in vitro and induce tumor growth (17, 28, 29, 96). Therefore, it was of interest to assess if the inhibition of HSV replication with acyclovir in combination with TPA treatment would induce tumors. However, acyclovir was not shown to

enhance tumor development when the substance was administered to HSV-1 infected mice. This indicates that there are more complex interactions between tobacco chemicals and HSV in tumor development than only inhibition of the virus replication.

The hypothesis that specific interactions between tobacco chemicals and HSV are of importance for the tumorogenic properties of these agents is sustained by the fact that we have recently found that diploid CHEF 18 cells, that are non permissive for HSV, when inoculated with HSV and treated with snuff extract during the first four passages have acquired the ability to grow in soft agar as opposed to cells exposed to HSV only (unpublished data). Interestingly, in repeated experiments, the CHEF cells did not survive treatment with snuff only.

Although it was shown (V) that snuff extract, at dilution 1:5, exerted toxic effects on cell growth and cellular protein synthesis in vitro, it had no detectable toxic effects at lower concentrations. This suggests that the inhibitory effect on HSV replication is a specific interaction with viral replication and is not secondary to toxic effects on cellular functions. This was further supported by the finding that the effect of snuff extract on HSV replication was shown to be dependent on the multiplicity of infection (V).

Neither anabasine nor the carcinogenic tobacco specific nitroseamines NNN and NNK had any significant effect on HSV replication when administered in non toxic doses. Furthermore, the concentrations of anabasine, NNN, and NNK in snuff extract are far below the levels at which they are toxic to cells or interferes with viral replication. However, this does not exclude that anabasine, NNN, and NNK contribute to the effect of snuff extract on HSV replication.

Nicotine was toxic to GMK cells at 1.0 mg/ml, but not at 0.5 mg/ml. The inhibitory effect on the HSV replication of nicotine at the latter concentration was significant. Although nicotine had an effect on HSV replication, it does not seem to solely account for the inhibitory effects of snuff extracts at dilutions 1:5 and 1:25. We have also recently found that the inhibitory effect of nicotine on HSV replication in primary human fibroblasts is counteracted by the addition of the nicotine receptor antagonist hexametonium (unpublished).

The tobacco induced inhibition of HSV replication has been reported to be at an early stage in the virus' replication cycle, i.e. before or at the level of the DNA replication (52, 80, 110). This is supported by the effects of snuff extract on HSV protein

synthesis in infected cells presented here. The increased production or accumulation of Á-proteins and the decreased production of Ó-proteins, in the presence of tobacco extracts, suggest that the block in the infectious cycle is temporally located between the production of these two groups of proteins at the level of ß-protein synthesis. The increased production of ICP 6 and ICP 8 and the probably decreased production of thymidine kinase and DNA polymerase indicate a more exact localisation of the block before synthesis of $ß_2$-proteins but after $ß_1$-proteins.

The mechanism underlying the block in virus replication is unclear. One possible explanation is that snuff extract blocks viral DNA replication as such. Stich et al. (80) and Oh et al. (110) found no effect on the synthesis of α- or ß-proteins when HSV infected vero-cells were exposed to snuff extracts or smoked tobacco tar, but a significant reduction of τ-protein synthesis. They concluded that the reduction of τ-protein synthesis was secondary to the supression of DNA-synthesis. The results of this study (IV) disprove that tobacco chemicals acts solely at the level of DNA synthesis in HSV replication.

Another possible mechanism is that snuff extract interferes with the virus host shut-off function. Both the finding of prolonged synthesis of ICP 4 and ICP 27 and of cellular actin in the infected cells exposed to snuff extract support this. The HSV host shut-off can be divided into two phases, one primary mediated by a virion component, which does not require the synthesis of viral DNA, and a late phase which reduces the remaining levels of host protein synthesis (35, 63, 89). The latter requires the expression of viral genes. Both phases are reported to be coded for by the same gene, mapping between 0.602 and 0.606 encoding for a 58 kilodalton protein. The finding that snuff extract administration induces a prolonged synthesis of cellular actin in HSV infected cells (V) might also suggest an interaction with the virus host shut off.

It is also possible that substances in snuff extract interact with α-proteins. Characterization of temperature-sensitive and deletion mutants has revealed that ICP 4 and ICP 27, and in resting cells ICP 22, are essential for productive HSV infection and their functional and physical properties have been extensively investigated (25, 91, 98). The α-proteins have been shown to act in trans to affect the transcription of specific subsets of viral genes. ß-protein synthesis is dependent only on the expression of functional α-proteins whereas the production of τ-proteins also requires various degrees of viral DNA synthesis for their production (Honess). 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 (84). The mechanism of the transport of ICP 4 from the cytoplasm to the nucleus and localization in the nucleus is not known. A possible effect of substances in tobacco could be interferencee with the compartmentalization of ICP 4, which - inter alia - would lead to a failiure in the autoregulative mechanisms of the ICP 4 synthesis (25).

The arrest of HSV replicative cycle at an early stage of infection with accumulation of immediate early proteins may have several implications. The immediate early proteins of HSV are potent trans-acting transcriptional activators (25, 91). ICP 4 is also a potent activator of HIV replication, a potent amplifier of the rabbit betaglobulin gene, and bovine papilloma virus DNA in human cells, and thus it has an effect also on alien DNA (2, 97, 105). Interestingly, patients with cancer in the head and neck had higher antibody titers to HSV-1 and in particular had a more frequent antibody reactivity against ICP 4 than controls (VI). Although there might be several explanations for this, one hypothesis is that tumor development in some cases is associated with a different course of an earlier HSV-1 infection having a more intense or prolonged antigen challenge. This does not exclude the possibility that tumors contain a partly expressed or an incomplete but active HSV genome continously stimulating the IgG response to HSV (18, 57, 61).

CONCLUSION

Non-smoked tobacco induces lesions in rat oral mucosa which are reversible after a limited time of exposure. It also has a weak carcinogenic effect, but it does not solely act as a promoter in tumor development. Exposition to the combination of HSV infection and tobacco results in a significant increase of tumor development in rats. This cannot only be explained by the fact that the inhibition of viral replication would allow HSV to express a possible carcinogenic effect since treatment of HSV-infected mice with acyclovir does not increase the tumor incidence. More likely complex interactions between tobacco chemicals and HSV are involved in the tumorigenic mechanisms. Non-smoked tobacco extract interferes with the replicative cycle of HSV at an early level of the infection. Cellular functions, as monitored by actin synthesis, are protected in HSV infected cells in the presence of tobacco extracts. Immediate early (α-) and some early (β-) proteins are accumulated in HSV infected cells when exposed to non-smoked tobacco. The high frequency of antibody reactivity to the HSV α-protein ICP 4 in patients with head and neck cancer, who are predominantly smokers, supports that the accumulation of immediate early proteins observed in HSV infected cells - when exposed to tobacco extracts - may be of relevance for cancer development in tobacco users.

ACKNOWLEDGMENTS

I should like to express my gratitude to

Anders Vahlne, who has been my teacher in the field of virology, for scientific guidance, constructive advice and generous support;

Jan Hirsch, for the initiation of this study, stimulating discussions, encouragement, and enthusiasm;

Sonny L. Johansson, for having introduced me into this field of study, continous support, fruitful collaboration, constructive critisism and friendly hospitality;

Erik Lycke, head of the Department of Clinical Virology, for creating a stimulating atmosphere at the institution, and for his support, and personal interest in my work;

Staffan Edström, for stimulating collaboration;

Simon Gronowitz, for valuable advice and performing the thymidine kinase and DNA-polymerase assays;

Bo Svennerholm, for pleasant collaboration;

The staff at the Department of Virology, for friendship, support, valuable help and good advice;

Lena Brändén-Hansson, for excellent technical assistance;

Hans Jansson, for help with the illustrations.

This investigation was supported by grants from the Faculty of Medicine, University of Göteborg, the Swedish Cancer Society, Grant No. 2555, the Göteborg Medical Society, Swedish Tobacco Company, Grant No. 8724, Assar Gabrielsson Foundation for Cancer Research, and the Foundations of Sahlgrens' Hospital.

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I

The reversibility of the snuff-induced lesion: an experimental study in the rat

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Snuff lesions were induced in 30 rats. Ten of the snuff-exposed rats were killed immediately after 13 months snuff exposure, as were the 10 control animals. Ten rats were killed 1 month and 10 rats 4 months after the snuff administration had ceased. The rats exposed to snuff for 13 months exhibited hyperplastic, hyperorthokeratotic epithelium with focal mild atypia, focal ulcerations and marked subepithelial fibrosis. These changes were markedly reduced or absent in rats exposed to snuff and killed after a snuff-free interval of 1 or 4 months. Similar differences between the test-groups were seen in the epithelium lining the gingival sulcus of the lower incisors. This area seems to be more sensitive to chemical exposure than the oral mucosa proper as more severe microscopical changes were seen here. Snuff exposure results in the development of a hyperplastic, reactive, reversible lesion of the oral mucosa, suggesting that snuff predominantly has promoting activity when administered for a relatively short interval of time.

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Accepted for publication October 29, 1986.

The health consequences of snuff use have been revieved and discussed in detail (1–4). At present, a consensus seems to have been reached acknowledging snuff as an oral carcinogenic agent when used as in North America and western Europe. Snuff-taking is known to induce characteristic lesions in the oral mucous membrane at the site of application of the quid (5–7). These lesions have generally been looked upon as reversible (8). However, this assumption has never been subjected to detailed investigation. This study was designed to evaluate the reversibility of snuff-induced lesions using a well established rat model (9).

Material and methods

Animals

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

Animal model

The rats were anaesthetised by intraperitoneal injection of pentobarbitone sodium (60 mg/ml, ACO AB, Solna, Sweden). The dose used was 35 mg/kg body weight. To minimise peroperative bleeding, 0.5 ml of a local anaesthetic (Xylocaine-Adrenaline ® 20 mg/ml + 12.5 µg, Astra Läkemedel Södertälje AB, Sweden) was infiltrated in the submucosa of the lower lip. The mucous membrane of the lip was then excised from the lateral to the lower incisors to 3 mm dorsal to the midline of the lip on both sides in a width of about 2 mm. The 2 wound surfaces thereby created were sutured together. This procedure resulted in a canal covered with mucous membrane. Two teflon plates were applied, one on each side of the lip, with stainless steel wire pulled through the plate-lip-plate. This stabilised and protected the lip during the healing phase for 10 days, after which the plates and sutures were removed (9).

Snuff

A commercially available Swedish brand was used in the study (kindly supplied fresh by Svenska Tobaks AB, Sweden).

Morphological method

Histological examination was performed on the lip, gingival epithelium of the lower incisors (crevicular epithelium), tongue and buccal mucosa. All specimens were fixed in 4% buffered neutral formalin solution and processed and stained by routine methods (H&E, and Weigert van Gieson).

Experimental design

Forty rats were operated on to create the test canal in the lower lip. The animals were then divided into 3 testgoups and one control group. Each group comprised 10 animals. All rats in the test-groups were given approximately 0.2 g of snuff at 8:00 and at 17:00 5 days per week for 13 months. The estimated average length of daily exposure to snuff was 12 h (9). The control animals (n = 10) underwent the

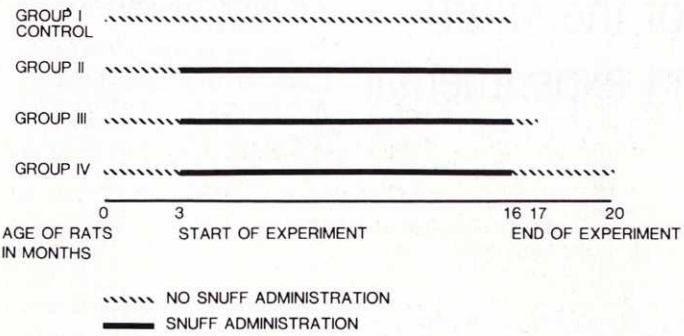

Fig. 1. Experimental design.

same surgical procedure but did not receive snuff. These rats were killed after 13 months. The rats in the first test-group were killed after 13 months of snuff exposure. The rats in the second test-group were killed 1 month after and those in the third test-group 4 months after the snuff administration had ceased. The experimental design is shown in Fig. 1.

Results

The lips of the control animals mostly showed slightly hyperplastic epithelium (90%) * with thickening of both the stratum ganulosum and the stratum spinosum. The surface was covered with a somewhat thickened orthokeratin layer (60%). The lumen of the test canals was partly filled with desquamated keratin (80%, Fig. 2). The rete ridges extending into the connective tissue were only slightly increased and the inflammatory reaction subepithelially in the connective tissue was mild (30%) or absent.

The squamous epithelium of the lips of the first test-group, exposed to snuff for 13 months, exhibited a generalised slight (40%) or moderate hyperplasia (60%). Hyperorthokeratosis was observed in all animals. In certain parts, marked hyperorthokeratosis was seen, while in others a looser type was seen, with focally vacuolated cells extending down into the stratum granulosum (50%). Slightly (80%) or moderatly (20%) acanthotic proliferations with development of marked rete pegs were noted (Fig. 3). The squamous epithelium showed mild focal atypia (40%) as well as focal ulcerations (20%) but the border between the stratum basale and the connective tissue was always well defined. The inflammatory reaction (mostly lymphocytic infiltrates) in the underlying connective tissue was slight (60%) or severe (40%), but above all a prominent fibrosis was noted (100%, Fig. 4).

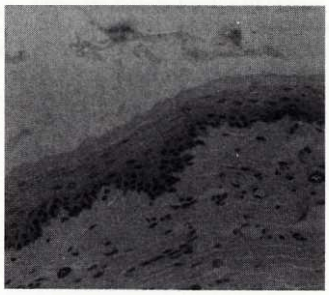

Fig. 2. Light-microscopic appearance of the test canal in a control rat at 13 months. Note the slightly hyperplastic epithelium with orthokeratosis and mild inflammatory reaction in the connective tissue (H&E, ×250).

In the 2 test-groups exposed to snuff and then killed after snuff-free intervals of 1 and 4 months respectively, histopathological changes in the test canals were less prominent. Thus, in comparison with the first test-group, only one rat in each test-group (10%) exhibited ulcerations. The lesions were more atrophic after a snuff-free interval of 1 month (1-month group, 30%) and 4 months (4-month group, 70%), with slight (4-month group, 30%) or no acanthosis (1-month group). The inflammatory reaction was slight (40%) or absent in both these groups (Fig. 5). Mild atypia of the squamous epithelium was only seen in one rat (in the 4-month group). In 60% of the rats with a snuff-free interval of 1 month and in all rats with a 4-month' snuff-free interval, severe subepithelial fibrosis was observed.

The appearance of the squamous epithelium of the tongues of rats killed immediately after 13 months of snuff exposure did not differ from that of the controls.

The squamous epithelium of the tongue and buccal mucosa in rats exposed to snuff for 13 months and killed 1 and 4 months thereafter was mildly hyperplastic (30% 1-month group, 90% 4-month group) and hyperkeratotic (30% 1 month and 70% 4 months) with a mild inflammatory reaction in the subepithelium tissue (30%).

The epithelium of the buccal mucosa was keratinised, with slight or moderate hyperplasia and slight acanthosis in all rats exposed to snuff for 13 months.

Moderate or severe hyperplasia with increased keratinisation was seen in the epithelium lining the gingival sulcus in rats exposed to snuff for 13 months and killed immediately (50%) or after one month (60%, Fig. 6). Focal resorption of the marginal bone plate buccally to the lower incisors was also noted in 2 cases, one in each group. Apart from these findings, the majority of the specimens exhibited atrophy and focal ulcerations (70%) of the gingival sulcus epithelium.

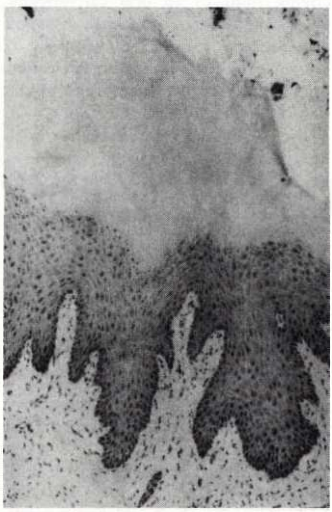

Fig. 3. Light-microscopic appearance of the test canal in a rat exposed to snuff for 13 months. Note the general moderate hyperplasia with acanthosis and hyperorthokeratosis (H&E, ×250).

* The figures in parentheses represent the percentage of animals in the group exhibiting the particular histologic feature described.

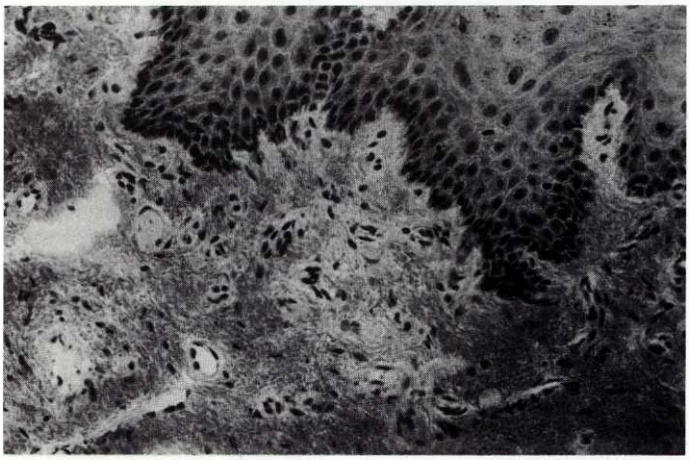

Fig. 4. Light-microscopic appearance of the test canal in a rat exposed to snuff for 13 months. Note the prominent fibrosis in the connective tissue and the mild inflammatory reaction (H&E, ×400).

The rats killed 4 months after termination of the snuff exposure, exhibited only slightly hyperplastic epithelium of the gingival sulcus (70%), with little or no keratinisation. The epithelial atrophy was less (30%) and only occasional ulcerations were seen.

The light microscopic appearance of the squamous epithelium of the buccal mucosa and the gingival sulcus of the control animals did not show any noteworthy pathological changes.

Discussion

All rats exposed to snuff for 13 months and then killed immediately exhibited lesions of the lip and oral cavity which were compatible with those described earlier (9, 10). The results of this study show that the described changes in the test canal after 13 months of snuff exposure followed by a snuff-free interval of 1–4 months are reversible to a certain extent. However, the squamous epithelium of the lips was atrophic and a few ulcerations were also seen after a snuff-free interval of 1–4 months. The subepithelial connective tissue exhibited extensive fibrosis.

The epithelium of the gingival sulcus of the lower incisors (crevicular epithelium) appeared to be more sensitive to snuff exposure than that of the tongue and buccal mucosa. This is probably due to the short distance between the test canal and the incisors. Furthermore, it seems likely that snuff is more or less constantly retained in the gingival sulcus, resulting in a longer exposure time in comparison with the buccal mucosa and tongue.

The gingival sulcus is covered with a thin unkeratinised epithelium, which may be more sensitive to snuff than the other locations, and this might explain the more pronounced microscopical changes found here.

Based on its content of tobacco specific nitrosamines – TSNA-snuff must be regarded as a carcinogenic agent (2). However, at the level present in snuff, especially in combination with a relatively short exposure time, the TSNA may predominantly act as promotors, resulting in proliferative lesions which to a large extent healed within 4 months after cessation of the snuff exposure.

We have previously reported on three snuff-induced intraoral squamous cell carcinomas using the same experimental model (11).

One tumour was detected after 9 months of snuff exposure and the other tumours were seen after 18 months of exposure to snuff and exposure to infectious Herpes simplex virus Type 1 (HSV-1, 12). All 3 tumours were located in close contact with the alveolar process. These tumours most likely originated from the gingival sulcus epithelium and not from the squamous epithelium of the test canal in the lip. This indicates that the squamous epithelium of the lip is rather resistant to exposure to snuff and chemicals in snuff such as the TSNA. The gingival sulcus area has also earlier been reported to be sensitive to effects of carcinogenic substances such as 4-nitroquinoline N-oxide, (4-NQO, 13).

It has been reported that squamous cell carcinoma may develop in the rat palate, even after as few as 3–6 applications of 4-NQO, if the latency time is sufficiently long (14). This suggests that the snuff-free interval before terminating this experiment may have been too short to result in tumour induction, or rather that snuff alone is a promotor or weak carcinogen requiring a much longer exposure time for tumour induction.

Although the lesions found in the test canal were found to be reversible to a certain extent, the canal serves as an excellent reservoir for the snuff. From this reservoir, snuff and products of snuff spill into the oral cavity for many hours. These substances may af-

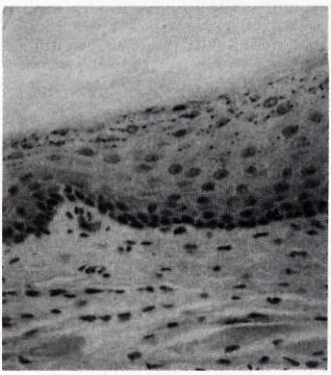

Fig. 5. Light-microscopic appearance of the test canal in a rat exposed to snuff for 13 months followed by a snuff-free interval of 4 months. Note the rather atrophic squamous epithelium in comparison with rats exposed to snuff for 13 months (Fig. 4, H&E, ×400).

Fig. 6. Light-microscopic appearance of the epithelium lining the gingival sulcus in a rat exposed to snuff for 13 months. Note the moderate epithelial hyperplasia, increased keratinisation and subepithelial inflammation (H&E, ×250).

fect different organs throughout the body since they diffuse into the bloodstream and are excreted into the saliva and urine (2). The TSNA and other related substances in snuff may also interact with other substances such as ethanol (15) or HSV-1 as shown earlier (16). Further studies are under way to investigate these associations.

Acknowledgements – The authors thank Lena Brändén-Hansson for excellent technical assistance. This study was supported by Svenska Tobaks AB and the Swedish Dental Association.

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II

[CANCER RESEARCH 49, 3063–3069, June 1, 1989]

Snuff-induced Carcinogenesis: Effect of Snuff in Rats Initiated with 4-Nitroquinoline *N*-Oxide[1]

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ABSTRACT

A canal in the lower lip to function as a reservoir for snuff was surgically created in 150 male Sprague-Dawley rats. The animals were randomized into five groups of 30 each: Group I received snuff twice a day, 5 days a wk; Group II was painted with propylene glycol (solvent control) on the hard palate 3 times a wk during 4 wk; Group III underwent painting on the hard palate with 4-nitroquinoline *N*-oxide (4-NQO) dissolved in propylene glycol, 3 times a wk for 4 wk; Group IV received 4-NQO as in Group III followed by snuff application as in Group I; and Group V received a cotton pellet dipped in saline twice a day, 5 days a wk. Treatment continued for up to 108 wk. There was no significant difference in mean survival time between the groups. Squamous cell tumors of the lip, oral and nasal cavities, esophagus, and forestomach were seen only in Groups I, III, and IV. Nine tumors of these organs were found in Group I (six carcinomas and three papillomas), nine in Group III (seven carcinomas and two papillomas), and ten in Group IV (eight carcinomas and two papillomas). The difference between each of these groups and the control groups (II and V) with regard to tumor incidence is statistically significant ($P < 0.05$). In Group I, four oral cavity or lip carcinomas were found in 29 rats, a significant difference in relation to control rats ($P < 0.05$). In addition, hyperplastic lesions of the lip, palate, and forestomach were significantly more common in Groups I and IV compared with Groups II, III, and V. The study has shown that snuff and 4-NQO by themselves have the potential to induce malignant tumors. Initiation with 4-NQO followed by snuff did not significantly enhance tumor formation.

INTRODUCTION

The use of moist snuff has been increasing in western Europe and the United States during the last decade. Snuff dipping is a habit which is most common among Caucasian men. It is often falsely portrayed as a less health-threatening substitute for cigarette smoking, although the snuff habit has considerable health consequences. Epidemiological studies have demonstrated that both the general health and oral health are affected in snuff dippers (1–3). The placement of snuff results in characteristic mucosal lesions in both rats and humans. These lesions have been shown to be reversible in rats after discontinuation of snuff exposure (4).

The most serious complication associated with snuff dipping is the markedly increased risk of developing oral cancer, especially after a long-time exposure. Thus, Winn *et al.* (5) demonstrated that snuff exposure lasting for 4 decades or longer was associated with approximately 50 times increased risk of developing squamous carcinoma in the oral cavity. The International Agency on Research on Cancer and the NIH have stated that there is sufficient evidence to regard snuff as an oral carcinogenic agent when used as in North America and western Europe (6, 7). It has been further stated that, in contrast to the human situation, sufficient evidence to support carcinogenicity of snuff in experimental animals is lacking (6). To some extent this may be related to the lack of a suitable animal model. Such a model, which allows long-time administration of snuff in a surgically created canal in the lower lip of the rat, has been developed (8). In this model oral tumors have been induced by administration of snuff alone (9, 10).

The development of cancer is regarded as a multistep process which can be divided into two major events, initiation and promotion (11). The findings of hyperplastic reversible lesions induced by snuff (4) may lend support to snuff functioning as a tumor promoter in the oral cavity. However, since snuff contains more than 3000 chemical substances, including some 20 tobacco-specific, volatile, and nonvolatile *N*-nitrosamines, some carcinogenic volatile aldehydes, polycyclic aromatic hydrocarbons, and Po-210, it is of considerable interest to evaluate the influence of snuff in both stages of cancer development (12, 13).

Attempts to chemically induce malignant tumors of the oral squamous epithelium in rats were unsuccessful until Fujino *et al.* (14) in 1965 introduced the water-soluble carcinogen 4-NQO.[3] The carcinogenic potential of 4-NQO is now well documented, and it has been shown to induce both oral and squamous cell carcinomas as well as spindle cell sarcomas in various rodent species (15–17).

The aim of the present investigation was to evaluate the tumor-promoting effects of snuff in rats initiated with a subcarcinogenic dose of 4-NQO as well as to determine the effects of long-term administration of snuff in male Sprague-Dawley rats.

MATERIALS AND METHODS

Animals

One-hundred fifty, 6-wk-old male Sprague-Dawley rats (Charles River Company, Portage, MI) were used. The rats were kept in quarantine for 2 wk and were randomized into 5 groups of 30 rats. They were kept in plastic cages with hardwood bedding, 5 rats in each cage. They were fed a standard pelleted diet (Prolab 3000; Agway, Inc., St. Mary, OH) and tap water *ad libitum*. Temperature was kept constant between 21 and 23°C, and the relative humidity was 50 ± 20%. They were exposed to 12 h of light and 12 h of darkness. A canal was surgically created in the lower lip according to the method described by Hirsch and Thilander (8). The rats were operated on when they were 8 to 9 wk old and weighed 250 to 300 g. After 3 to 4 wk of healing, the rats were subjected to the experimental regimens.

Snuff

A commercially available United States brand purchased every other month on the open market in Omaha, NE, was used in the study. The snuff was kept at 4°C. The snuff was applied in the test canal, which

Received 12/12/88; revised 3/6/89; accepted 3/8/89.

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[1] This study was supported in part by funds from the Department of Pathology and Microbiology, University of Nebraska Medical Center; by NIH Research Grant CA36727; and by funds from the Smokeless Tobacco Research Council (Grant 0144-1).

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[3] The abbreviations used are: 4-NQO, 4-nitroquinoline-*N*-oxide; NNN, *N'*-nitrosonornicotine; NAT, *N'*-nitrosoanatabine; NNK, *N*-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)butanone; NPYR, *N*-nitrosopyrrolidine; TSNA, tobacco-specific *N*-nitrosamines; GLC, gas-liquid chromatography; TEA, thermal energy analyzer.

was completely filled 2 times a day by use of a spatula. At least 100 mg were administered with each application. Immediately before the afternoon applications, the old snuff from the morning was removed. The average exposure time was 8 to 16 h daily (10).

4-NQO

In order to initiate the squamous epithelium of the hard palate, 4-NQO (Sigma Chemical Co., St. Louis, MO) was dissolved in propylene glycol to a concentration of 0.5%. At each application, approximately 0.13 mg of 4-NQO were applied to the palatal mucosa.

Analysis of Tobacco-specific *N*-Nitrosamines

The TSNA, NNN, NAT, and NNK, were gifts from Dr. J. D. Adams, Naylor Dana Institute for Disease Prevention, American Health Foundation, Valhalla, NY. Stock and standard solutions of the *N*-nitrosamines were prepared in chloroform. Dichloromethane (analytical grade) was obtained from Riedel de Haen AG (Seelze-Hannover, Federal Republic of Germany), and chloroform (high-performance liquid chromatography grade) was purchased from Fisons Scientific Apparatus (Loughborough, England). Kieselguhr (Extrelut; E. Merck AG, Darmstadt, Federal Republic of Germany) was dried overnight at 160°C prior to use and stored at the same temperature. The organic solvents were checked to ensure the absence of substances that could interfere with the analysis for *N*-nitrosamines. Three-g samples of snuff were suspended in 20 ml of dichloromethane in a flask. After allowing to stand for 30 min at room temperature, the mixture was placed on an Extrelut column (15 cm long, 2-cm inner diameter), and after 15 min the column was eluted with dichloromethane (4 x 25 ml). The eluate was concentrated to about 1 ml in a water bath at 55°C. The extract was transferred to a vial and diluted to 5.0 ml with chloroform.

Analyses were carried out using isothermal GLC (Model 2700; Varian, Palo Alto, CA), interfaced with a TEA (Model 502; Thermo Electron Corp., Waltham, MA). The furnace was removed from the TEA and connected to the GLC column via a 5.5-cm-long glass tube. For detection and quantification, 5.0-μl portions were analyzed against external standards by injection into a 1.8- x 1.9-mm (inside diameter) glass column containing 10% UCW-982 on Chromosorb W, AW-DMCS, 80/100 mesh. The GLC-TEA conditions were: column temperature, 200°C; injector temperature, 250°C; helium carrier-gas flow, ~28 ml/min (1.8-m column); furnace temperature, 475°C, oxygen flow, ~10 ml/min; and vacuum pressure, ~0.6 mm of Hg. A CRT gas stream filter (Thermo Electron Corp.) was used. The detection limits of the method were 0.01 to 0.02 mg of tobacco-specific *N*-nitrosamines/kg wet weight of snuff.

Analysis of Volatile *N*-Nitrosamines

The 5-ml extract from the analysis of TSNA above was concentrated to about 0.5 ml in a water bath at 70°C. The final volume was measured with a 1000-μl Hamilton syringe.

Analyses were carried out by injection of 5-μl portions into a GLC-TEA equipped with a 1.8-m x 1.9-mm (inner diameter) glass column containing 20% Carbowax 20M and 2% KOH on Chromosorb W, AW-DMCS, 80/100 mesh. The GLC-TEA conditions were: column temperature, 160°C; injector temperature, 200°C; helium carrier-gas flow, ~27 ml/min; furnace temperature, 475°C; oxygen flow, ~10 ml/min; vacuum pressure, ~0.7 mm of Hg. A CRT gas stream filter was used.

The detection limits of the method were 0.5 to 1 μg of volatile nitrosamines/kg wet weight of snuff.

Analysis was performed on every tenth box of snuff, and altogether 28 boxes were analyzed. The levels of tobacco-specific and volatile *N*-nitrosamines are given in Table 1.

Experimental Design

The rats were divided into 5 groups, each containing 30 rats. All rats were operated on as described above, and the experimental treatment began 1 mo after the canal in the lower lips was surgically created and the rats were 3 mo old (summarized in Table 2). This was considered time zero of the experiment. In 3 rats the canals were not suitable for snuff application, and these rats were excluded from the experiment. The following groups were used in the study.

Table 1 *Isolated amounts of TSNA and volatile N-nitrosamines in the snuff used for the long-term bioassay*

The values are based on wet weight (moisture content about 50%).

TSNA content (mg/kg)		Volatile *n*-nitrosamine content (μg/kg)	
NNN	NAT	NNK	NPYR
5.14 ± 1.39[a]	5.09 ± 1.02	0.89 ± 0.12	22 ± 7.0

[a] Mean ± SD.

Table 2 *Experimental design*

Group	Treatment		No. of wk
I	Surgery	Snuff	104
II	Surgery	Propylene glycol	4
III	Surgery	4-NQO	4
IV	Surgery	4-NQO + snuff	4 + 104
V	Surgery	Cotton pellet	104

Group I (30 Rats). Snuff was applied in the experimental canal 2 times daily as described above beginning at wk 4 of the experiment. This was repeated 5 days a wk for up to 104 wk.

Group II (29 Rats). Propylene glycol was applied to the palate 3 times weekly with a sable hair brush (No. 2) with a one-stroke painting from the soft palate to papilla incisiva. No anesthesia was used. The treatment was applied for the first 4 wk of the experiment, followed by no further treatment for the remaining 104 wk of the experiment.

Group III (29 Rats). 4-NQO dissolved in propylene glycol was applied as described above for Group II.

Group IV (30 Rats). 4-NQO was applied identically to the rats of Group III for the first 4 wk, and the rats were then treated with snuff identically to the animals of Group I for the following 104 wk.

Group V (29 Rats). They were operated on and received a cotton pellet dipped in physiological saline twice a day, 5 days a wk for the last 104 wk of the experiment.

All remaining rats were terminated at 108 wk after the start of the different treatment regimens.

Morphological Methods

All animals underwent complete autopsy for the recording of tumors and other pathological lesions. Specimens from the lip, test canal, palate, oral and nasal cavities, lungs, heart, liver, esophagus, forestomach, glandular stomach, kidneys, urinary bladder, and other grossly abnormal tissues were taken for light microscopic examination. Tissue specimens were fixed in 4% neutral buffered formalin solution, embedded in paraffin, sectioned, and stained by hematoxylin-eosin. Immunochemical staining with antibodies against keratin (MAK-6) and vimentin was performed on selected cases.

Statistical Methods

Statistical significance was calculated by Fisher's exact test (18). A P value of < 0.05 was regarded as statistically significant.

RESULTS

Two rats in Group IV and one rat each in Groups I and II died during the experiment and were not suitable for histopathological evaluation due to severe autolysis and/or extensive cannibalism. The mean survival time in the different groups is shown in Table 3. There was no significant difference in survival time between the groups.

The average body weight of the rats at the beginning of the experimental regimens was approximately 375 g in all groups. The body weight curve of the different groups during the experiment is shown in Fig. 1. As shown in this figure, the snuff-treated groups had a slower weight gain than the groups which did not receive snuff. The difference in body weight

Table 3 *Mean survival time in the different groups (wk)*

Group	(n)[a]	Mean survival	
		Wk	Range
I	29	106	68–120
II	28	102	71–119
III	29	102	76–119
IV	28	97	53–120
V	29	100	56–119

[a] Number of rats available for histopathological evaluation.

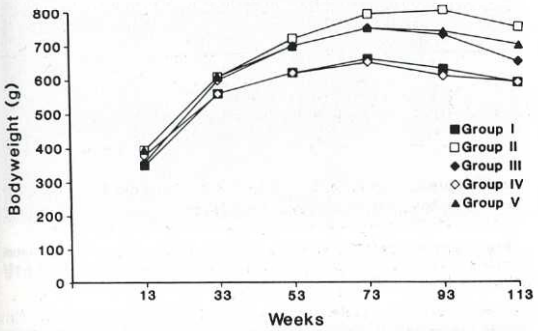

Fig. 1. The mean body weight of the different groups.

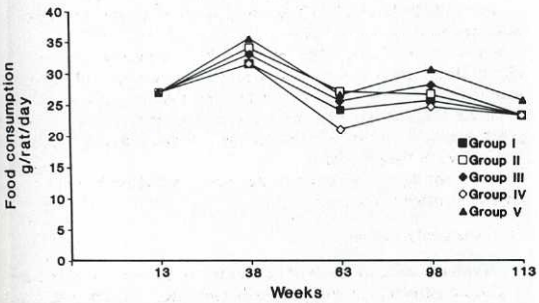

Fig. 2. Daily food consumption per rat in the different groups.

between the snuff-treated groups (I and IV) and the other groups (I, III, and V) was 100 g after 40 wk and remained at this level throughout the experiment (statistically significant at $P < 0.05$).

The food consumption during the experiment is given in Fig. 2. In the beginning of the experimental treatment, the food consumption was the same in all groups, on the average, 27 g per day. The differences in food consumption between the groups during the experiment were not statistically significant, but throughout the experiment, Groups I and IV consumed less food than the other groups. The average daily water consumption did not significantly differ between the groups, but after an initial decrease, it increased in all groups until the end of the experiment (Fig. 3).

The incidence of tumors and hyperplastic (possibly preneoplastic) lesions is given in Table 4. The tumor data are basically expressed per tumor-bearing animal. However, 2 rats in each of Groups I, III, and IV had 2 primary tumors, but only one of them (in Group III) had 2 squamous cell carcinomas (tongue and forestomach). Squamous tumors (papilloma or carcinoma) of the lip, oral cavity (tongue, hard palate), nasal cavity, esophagus, and forestomach were exclusively seen in rats treated with snuff only, 4-NQO only, or 4-NQO followed by snuff. In Group I (snuff only), there were 6 squamous cell carcinomas and 3

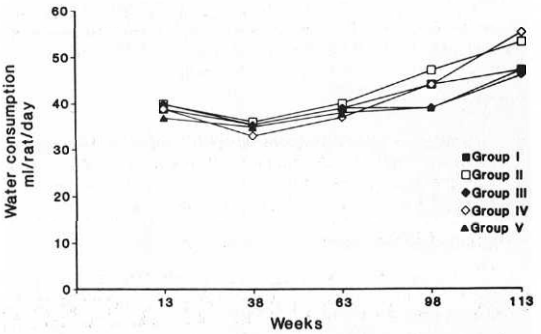

Fig. 3. Daily water consumption in the different groups.

papillomas; in Group III (4-NQO only), there were 7 carcinomas in 6 rats and 2 papillomas; and in Group IV (4-NQO followed by snuff), there were 8 carcinomas and 2 papillomas (Figs. 4 to 6). The differences in incidence of squamous cell tumors between Groups I, III, and IV *versus* Groups II and V are statistically significant ($P < 0.01$). Even if limited to malignant squamous cell tumors, the difference is statistically significant ($P < 0.05$). In addition, hyperplastic and/or dysplastic lesions of the squamous epithelium in the lip, hard palate, and forestomach were significantly more common in Groups I and IV (Table 4; Fig. 7). Furthermore, there were two spindle cell sarcomas of the lip in Group I and three in Group IV (Figs. 8 to 11). The tumor cells in these rats were negative for keratin and positive for vimentin by immunohistochemical staining. These tumors grew rapidly, and one metastasized to the lungs (Group IV). Two rats in Group I and one in Group IV had moderately well-differentiated hepatocellular carcinomas.

The overall tumor incidence was highest in Groups I and IV, 23 tumors in 21 rats being present in Group I and 22 in 20 rats in Group IV (Table 4). In the group of rats treated with 4-NQO only, 13 tumors in 11 rats were found; in the propylene glycol-treated group, 5 tumors; and in the control group, 3 tumors. The difference in total tumor incidence calculated per tumor-bearing animals between Groups I and IV as compared with groups II and V is statistically significant ($P < 0.01$), as well as the difference in total tumor incidence between Groups I and IV *versus* Group III ($P < 0.05$).

Marked inflammatory changes with foreign body giant cell reaction were seen in 92% of the rats of Groups I and IV (Fig. 12). In contrast, only 20 to 30% of the rats in Groups II, III, and V had inflammatory changes, and foreign body granulomas were virtually absent. Severe fibrosis of the lip was seen in almost all of the rats of Groups I and IV. Less pronounced fibrosis was seen in the lips of Groups II, III, and V.

DISCUSSION

Chronic snuff exposure, with or without preceding 4-NQO treatment, did not significantly influence the mean survival time of the rats (Table 3). The mean body weight of the rats (Fig. 1) was almost identical in all groups at the beginning of the experiment. However, after 40 wk of snuff administration, the body weight was approximately 100 g lower in the snuff-treated groups (I and IV) as compared to the other groups (Groups II, III, and V). From 40 wk on, this difference remained at the same level until the end of the experiment. The difference in weight between the snuff-exposed rats and controlled rats

Table 4 *Incidence and distribution of tumors and preneoplastic lesions in the different groups of male Sprague-Dawley rats*

Type of lesion and location	Group I (29)[a]	II (28)	III (29)	IV (28)	V (29)
Squamous cell carcinoma					
Lip	1[b]				
Hard palate	2		2[c]	4[d]	
Tongue			2[e]	1[f]	
Nasal cavity	1			1	
Esophagus			1		
Forestomach	1		2	2	
Squamous cell carcinoma *in situ*					
Lip				1	
Hard palate	1				
Squamous cell papilloma					
Lip	1				
Hard palate	1				
Tongue			2	1	
Nasal cavity	1				
Hepatoma	2[g]			1	
Adenocarcinoma					
Breast					1
Kidney					
Renal pelvic tumor	1				
Wilms' tumor					1
Testis					
Leydig cell tumor				1	1
Pituitary gland					
Adenoma	1				
Malignant lymphoma	4		1	1	
Malignant fibrous histiocytoma					
Skin-subcutis	2	1	1		
Sarcoma undifferentiated					
Lip	2			3	
Skin			2	1	
Neurofibrosarcoma					
Skin-subcutis	1	1			
Neurofibroma					
Skin-subcutis		2			
Fibroma					
Skin	1			1	
Squamous cell hyperplasia					
Lip, lip canal	24	6	4	25	10
Hard palate	18	7	7	14	2
Forestomach	18	4	6	18	1
Squamous cell dysplasia					
Lip, lip canal	10		4	12	
Hard palate	5			7	

[a] Numbers in parentheses, effective number of rats.
[b] This rat had both lip papilloma and neurofibrosarcoma of skin.
[c] One of these rats had both carcinoma of hard palate and skin sarcoma.
[d] One of these rats had both carcinoma of hard palate and sarcoma of lip.
[e] One of these rats had carcinoma of both tongue and forestomach.
[f] One of these rats had both carcinoma of tongue and malignant lymphoma.
[g] This rat had both hepatoma and malignant fibrous histiocytoma of skin.

was similar to what we found in an earlier study (10), which is in accordance with the results by Hecht *et al.* (9) who reported weight differences lasting through wk 84 of their study. After that time, no difference was recorded. They administered snuff only 5 times/wk as compared to 10 times in our study, and another commerical brand of snuff was used.[4] Ninety-two % of the rats in Groups I and IV had marked inflammatory changes with foreign body giant cell reaction, which may have been associated with soreness and pain in the lip preventing optimal

[4] D. Hoffmann, personal communication.

Fig. 4. Polypoid invasive moderately differentiated squamous cell carcinoma occurring at the transition between the hard palate and the nasal cavity in a rat exposed to snuff. H & E, × 33.

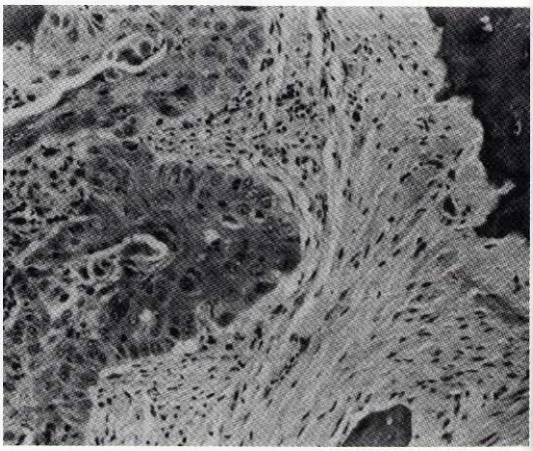

Fig. 5. Poorly differentiated squamous cell carcinoma of the hard palate invading bone in a Group I rat. H & E, × 132.

food intake. This is supported by the lower food consumption in the rats treated with snuff (Fig. 2). It is also known that snuff-treated rats have high blood concentrations of nicotine (8), resulting in an increased level of metabolism which also might have contributed to the lower weight gain of these rats.

The water consumption did not differ significantly among the groups. However, there was an increased water consumption among all rats towards the end of the experiment which may be related to the pronounced rat nephrosis present in the majority of rats at the time of sacrifice (19).

Snuff administration resulted in a high number of tumors overall. There were 23 tumors in Group I and 22 in Group IV, respectively, which is significantly higher than in the control Groups II and V or rats treated with 4-NQO only (Group III) (see Table 3). Squamous cell tumors (carcinoma and papilloma) of the lip, oral cavity, nasal cavity, esophagus, and forestomach were exclusively seen in rats treated with snuff only (Group I, nine tumors), 4-NQO (Group III, nine tumors), and 4-NQO

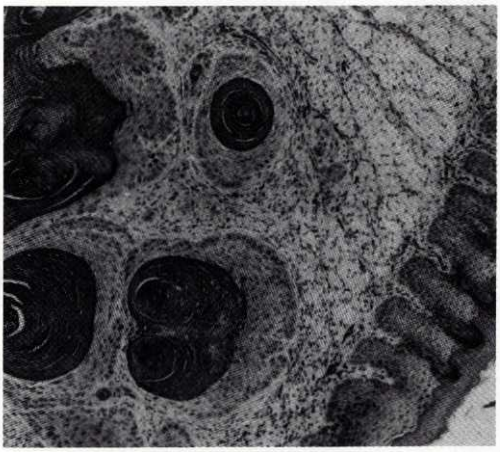

Fig. 6. Well-differentiated invasive squamous cell carcinoma of the tongue in a Group IV rat. H & E, × 96.

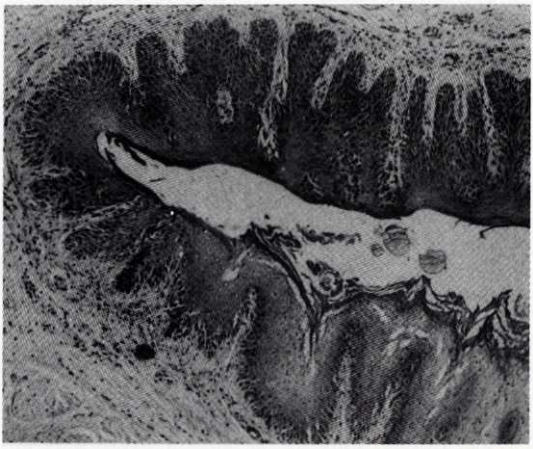

Fig. 8. Section of the lip canal in a rat exposed to snuff only. Note the marked diffuse hyperplasia and focal dysplasia of squamous epithelium. H & E, × 66.

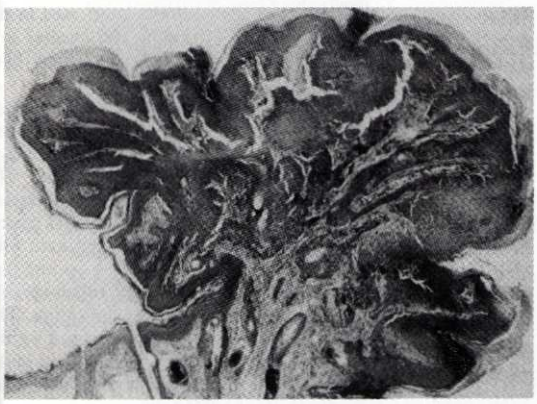

Fig. 7. Squamous cell papilloma of the lip in a snuff-exposed rat. H & E, × 33.

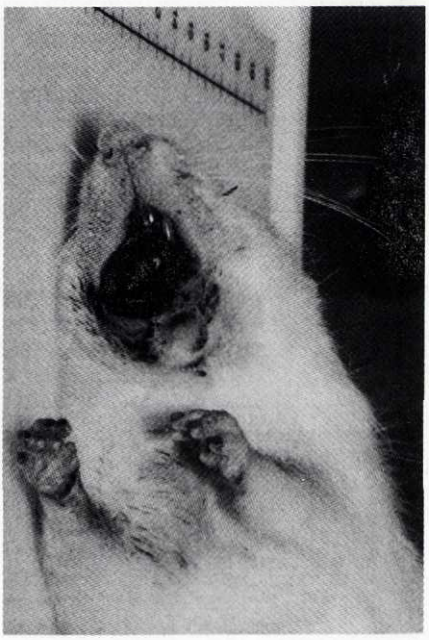

Fig. 9. Gross appearance of sarcoma of the lower lip in a rat exposed to snuff only.

followed by snuff (Group IV, ten tumors). Of these tumors, six in Group I, seven in Group III, and eight in Group IV were carcinomas. In contrast, no such tumors were encountered in the control groups (II and V). The difference in tumor incidence between Groups I and IV and Groups II and III is statistically significant ($P \leq 0.01$ and 0.05). Thus, the present study demonstrates that long-term snuff administration has the potential to induce malignant tumors. Wallenius and Lekholm (16) have shown that 100% of rats treated with 4-NQO for 6 to 8 mo developed oral squamous cell carcinomas. In this study, 14% of the rats exposed to snuff only developed intraoral carcinomas. Thus, snuff has a significantly lower level of carcinogenic activity than does 4-NQO. However, 10 weekly applications of snuff during a 2-yr period result in approximately the same incidence of intraoral cancers as 12 applications of 4-NQO administered over a 4-wk period followed by up to 104 wk of observation.

With regard to oral cancer, our figures are similar to the results obtained by Hecht *et al.* (9), although the number of oral tumors is somewhat higher in our study. This may be related to the fact that snuff was administered 10 times per wk in our study in comparison to 5 times per wk in their study and that we used another brand snuff.[4]

Also, TSNA were present in relatively high concentrations in our commercial snuff. The mean value of 28 samples of snuff was: NNN, 5.14 mg/kg; NNK, 0.89 mg/kg; and NAT, 5.09 mg/kg. NPYR was the only volatile *N*-nitrosamine present at detectable levels in all snuff samples (Table 1).

Our results show that snuff did not exert any promoting capability in the oral cavity or elsewhere when the rats were initiated with 4-NQO in the hard palate for 4 wk, since the number of tumors in Groups I and IV was virtually identical.

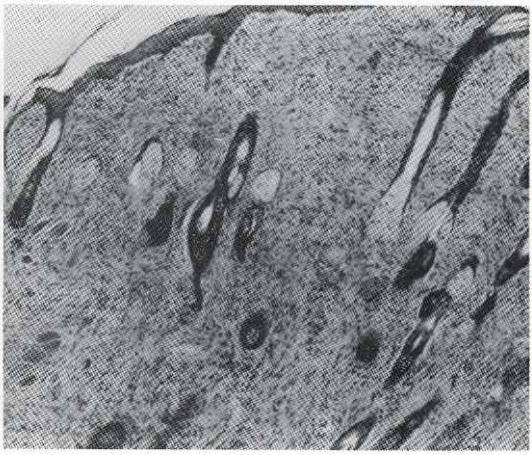

Fig. 10. Micrograph of the tumor in Fig. 9. H & E, × 66.

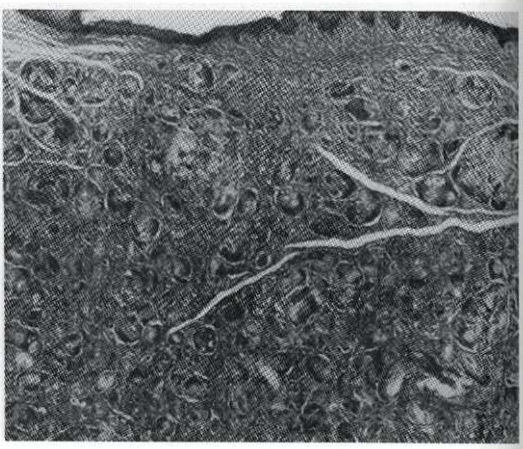

Fig. 12. Section of the lip canal in a snuff-exposed rat. Note the inflammatory response with extensive foreign body giant cell reaction. H & E, × 96.

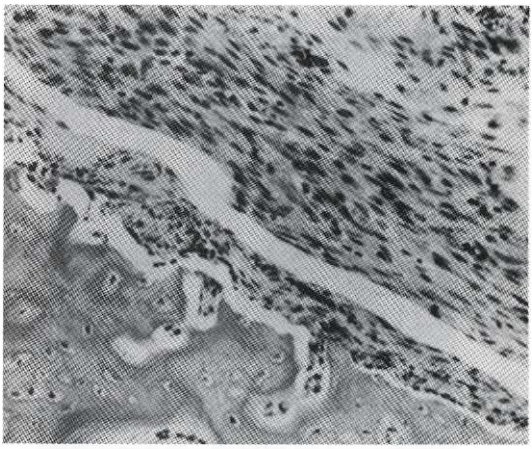

Fig. 11. Micrograph of the tumor in Fig. 9, showing bone invasion. H & E, × 132.

In a recently finished study, initiation was performed in the lip canal with 4-NQO.[5] No promoting effects of snuff in the lip or oral cavity were found, similar to the results of the present study.

NNK administration is strongly associated with the development of liver tumors. This compound, which is present in snuff, is likely to be responsible for the development of the three hepatomas in Groups I and IV. Snuff by itself can be carcinogenic for the lip, oral cavity, nasal cavity, and forestomach. This is probably due to its high concentration of TSNA, which have been shown to be organ-specific carcinogens to these organs (13). Other chemicals in snuff may also contribute to its inflammatory and neoplastic effects.

Five lip sarcomas were seen in Groups I and IV (Figs. 10 to 12). One of the sarcomas in Group IV metastasized to the lungs, which has not been reported previously in 4-NQO-induced sarcomas. Since no lip sarcomas were seen in Group III, it seems most likely that these tumors were related to snuff exposure rather than 4-NQO. United States snuff used in the present study was quite coarse and irritating and resulted in a significant inflammatory reaction (Fig. 12) which may have contributed to the development of these sarcomas.

Whether the difference in tumor incidences between Groups III and IV reflects a tumor-promoting effect of snuff is difficult to assess, but it seems unlikely since snuff by itself (Group I) induced as many tumors as 4-NQO followed by snuff. The results of this study indicate that snuff may exert tumorigenic effects in different organs of the rat (oral cavity, nasal cavity, esophagus, forestomach, and liver). This is in accordance with the hypothesis proposed by Hecht *et al.* (9). It is supposed that the high content of TSNA in snuff initiates organ-specific tumors, not only local tumors at the site of application. The epidemiological studies performed so far have focused on the relationship between snuff and oral cancer. The present study suggests that future epidemiological studies should evaluate the general tumorigenic effects of the snuff habit.

[5] P-A. Larsson *et al.*, unpublished observation.

ACKNOWLEDGMENTS

The authors thank Samuel M. Cohen, M.D., Ph.D., for his advice and comments. The authors also thank Claudia Borgeson, Lori Davis, Patrik L. Johansson, John Parr, Takao Sakata, Margaret St. John, and Scott Tibbels for excellent technical assistance as well as Shirley C. Murray for excellent secretarial help.

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III

Snuff tumorigenesis: effects of long-term snuff administration after initiation with 4-nitroquinoline-N-oxide and herpes simplex virus type 1

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Larsson P-A, Johansson SL, Vahlne A, Hirsch JM: Snuff tumorigenesis: effects of long-term snuff administration after initiation with 4-nitroquinoline-N-oxide and herpes simplex virus type 1. J Oral Pathol Med 1989; 18: 187–192.

The tumor promoting effects of snuff was studied in Lewis rats initiated with 4-nitroquinoline-N-oxide (4-NQO) and Sprague Dawley rats repeatedly inoculated with herpes simplex virus type 1 (HSV-1). The test substances were administered in a surgically created canal in the lower lips of the rats. There were 15 rats in each test group and 10 rats in the control group. In the groups treated with 4-NQO and 4-NQO + snuff, 8 and 12 tumors (5 and 9 malignant) were found, respectively. In the group subjected to HSV-1 only, 3 tumors were found (2 malignant), in the group subjected to snuff only, 4 tumors were found (3 malignant) and in the group subjected to the combination of HSV-1 and snuff, 13 tumors were found (7 malignant). In the control group only one malignancy was found. The study did not show any promoting effects of snuff in the oral cavity after initiation with 4-NQO. Neither was there any increase in the number of oral tumors in rats treated with HSV-1 and snuff. However, there was a marked increase in the number of malignant tumors outside the oral cavity in the group treated with HSV-1 and snuff, underlining the importance of interactions between these two agents in the development of malignant lesions.

Key words: herpes simplex; smokeless tobacco; snuff; tumorigenesis.

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Accepted for publication February 6, 1989.

Earlier studies have demonstrated that snuff dipping affects both general and oral health (1–3). Snuff dipping results in characteristic intraoral mucosal lesions, both in rats and humans. In rats these lesions have been shown to be reversible after discontinuation of snuff (4).

The most serious complication associated with snuff dipping is the markedly increased risk of developing oral cancer after long exposure. Thus, WINN *et al.* (5) demonstrated that snuff dipping is strongly associated with an increased risk of developing oral squamous cell carcinoma. In contrast to the human situation sufficient evidence to support carcinogenicity of snuff in experimental animal is lacking (6). To some extent this has earlier been related to the lack of a pertinent animal model. Such a model (7) has been developed, allowing life-long administration of snuff in a surgically created canal in the lower lip of the rat. Simulated snuff-dipping using this model have resulted in the development of oral tumors (8–10).

The development of cancer can be regarded as a two stage process, initiation and promotion (11). It has earlier been shown that 4-nitroquinoline-N-oxide (4-NQO) is a potent intraoral carcinogen. Thus 100% of animals painted in the hard palate for 6–8 months with 4-NQO developed carcinomas at the site of application (12). There is evidence supporting that a limited number of applications of 4-NQO is sufficient to initiate intraoral squamous cell carcinomas (13).

A large number of reports have associated herpes simplex virus type one (HSV-1) with leukoplakias, epithelial dysplasias and oral as well as cancers of the head and neck region (14, 15). HSV-1 is a ubiquitous human oral pathogen and approximately 70% of the adult population in the USA and western Europe is seropositive for HSV-1. Several previous reports have demonstrated that HSV-1 is capable of transforming cells in vitro (16, 17). A prerequisite for HSV to cause cell trans-

Table 1. Experimental protocol.

Group	Treatment		
I	Surgery	HSV-1	
II	Surgery		Snuff
III	Surgery	HSV-1	Snuff
IV	Surgery	4-NQO	
V	Surgery	4-NQO	Snuff
VI	Surgery		

formation is that virus induced cell lysis is prevented. In an earlier study we have demonstrated that snuff is capable of preventing HSV induced cell lysis (18), which may be one mechanism in snuff/HSV-1 tumorigenesis. Earlier studies have also shown that repeated infection with HSV-1 and simulated snuff dipping in rats and hamsters have resulted in induction of oral squamous cell carcinomas (10, 19).

The aim of the present study was to evaluate if snuff functions as a tumor promoter in rats initiated with 4-NQO or HSV-1. Using the earlier described model, rats were initiated with 4-NQO or inoculated with HSV-1 followed by life-long simulated snuff dipping in order to assess the tumor promoting effects.

Material and methods

Animals

Male, inbred Lewis rats (Groups IV & V) and Sprague Dawley rats (Groups I, II, III and, VI), purchased from Anticimex AB, Stockholm, were used. The rats were 3-months old with an avarage weight of 400 g when the experiment started. Three to four rats were kept in macrolon cages, (NO 3, Jacoby, Stockholm) with hardwood bedding. The animals were fed a standard pelleted diet (Astra Ewos AB, Södertälje), and tap water *ad libitum*. Temperature was kept constant between 21–23°C and the relative humidity 40%. Light followed diurnal rhythm and was never less than 8 h. The rats underwent a surgical procedure, described earlier, to create a canal in the lower lip which functioned as a reservoir for the test substances (7). The operation was followed by a 10-day healing phase, whereupon the experimental treatment began.

Snuff

A commercially available Swedish brand was used in the study. Analyses of the tobacco specific N-nitrosamines (TSNA) in this brand has been performed earlier. The average concentration of TSNA is: N-nitrosonornicotin 33 000 µg/kg, 4-(methylnitrosamino)-1(-3pyridyl)-1-butanon 4 600 µg/kg, N-nitrosoanatabin 40 000 µg/kg, and N-nitrosoanabasin 1900 µg/kg respectively. The snuff was applied in the test canal, 8 AM and 4:30 PM 5 days weekly by means of a plastic syringe. The test canal was completely filled which on the average meant the position of 200 mg of snuff. The average exposition time was 12 h per day (7).

4-nitroquinoline-N-oxide (4-NQO)

4-NQO was used as an initiator and purchased from Fluka Ag Buchs S.G. Switzerland. The chemical was dissolved in propyleneglycol to a concentration of 0.5%.

Virus

Herpes simplex virus, type 1, strain F (HSV-1) was used in this study. Virus infectivity was assayed by plaque titration on green monley kidney cells (GMK-AH1) and expressed as plaque forming units (pfu) per ml.

Experimental design

Eighty-five rats were divided into six groups. All rats were operated on as described above and the experimental treatment began after a healing phase of 10 days. The experimental design is summarized in Table 1.

Group I – comprising 15 Sprague-Dawley rats, received 0.5 ml of HSV-1 suspension, 2.5×10^7 pfu/ml, absorbed in a cotton swab. The swab was placed in the test canal in the lower lip after scarification with a 26 gauge needle. This was repeated once monthly until the animals were killed at the end of the experiment, thus mimicking a recurrent HSV-infection (10).

Group II – comprising 15 Sprague-Dawley rats received snuff five days weekly, as described above until killed.

Group III – comprising 15 Sprague-Dawley rats, was treated with HSV-1 identically to Group I, but the treatment also included snuff administration identical to Group II, except for 1 day monthly which was occupied by the virus inoculation.

Group IV – comprising 15 Lewis rats received 4-NQO once weekly during five consecutive weeks. 0.05 ml of the solution (0.25 mg 4-NQO) was absorbed in a cotton swab. During each application, the swab was put in the test canal 24 h and then removed.

Group V – comprising 15 Lewis rats, was treated with 4-NQO identical to Group IV for 5 weeks followed by snuff administration 5 days weekly, identically to Groups II and III.

Group VI – comprising 10 Sprague-Dawley rats served as a control group and was treated with propyleneglycol on a cotton swab once weekly during 5 wk.

Two rats in Group I died from encephalitis after 21 and 27 wk, respectively and were excluded from the study. Another 11 rats were found dead and suffered from pronounced autolysis and were therefore excluded from the experiment. The remaining rats constitute the effective number of experimental animals shown in Tables 2–4.

All animals were killed when moribund after 16–30 months by injection of phenobarbital followed by exsanguination until cardiac arrest.

Histopathologic methods

Following gross exterior inspection all animals underwent necropsy for the recording of tumors. Specimens from gingival sulcus, test canal, lower lip, lungs, heart, liver, forestomach, urinary bladder, spleen, brain and other grossly abnormal tissues were taken for light microscopy examination. Tissue specimens were fixed in 4% neutral buffered formalin solution, embedded in paraffin, sectioned and stained by routine methods, hematoxylin – eosin and according to Weigert van Gieson. The specimens were coded and evaluated independently by two of the authors (P-A L. and SL J). The preneoplastic and reactive changes of the oral cavity were classified in accordance with the criteria given in "Definitons of leukoplakia and related lesions: an aid to studies of oral precancer" (20). Rat nephrosis was in three grades,

Table 2. Mean survival time in the different groups.

Group	*n*	Wk	Range
I	12	85	54–102
II	13	86	70–94
III	15	89	76–102
IV	12	116	82–131
V	12	115	95–123
VI	8	90	61–102

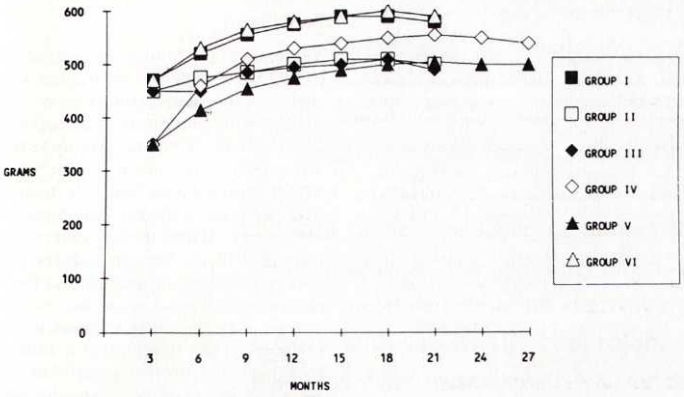

Fig. 1. Body weight curves for different groups.

as 1 (+), 2 (++), and 3 (+++) corresponding to mild, moderate, and severe.

Statistical methods

Statistical significance of differences in tumor incidence between the groups was calculated with Fisher's exact test. $P < 0.05$ was regarded statistically significant.

Results

General health effects

The mean survival time is given in Table 2. The Lewis rats (Groups IV and V) survived 6 months longer than the Sprague-Dawley rats. The mean body weight of the rats during the experiment is presented in Fig. 1. The weight of the Lewis rats at the start of the experiment was approximately 100 g less than the weight of the Sprague-Dawley rats. This difference diminished slightly but persisted throughout the experiment. Groups II, III, and V (snuff treated) had a slower weight gain than Groups I, IV, and VI. The difference in weight between snuff treated and not snuff treated groups was also statistically significant at 20 months age. Brain specimens from the two rats in Group I who died after 21 and 27 wk showed acute encephalitis but brain specimens from all other animals were normal by histologic examination.

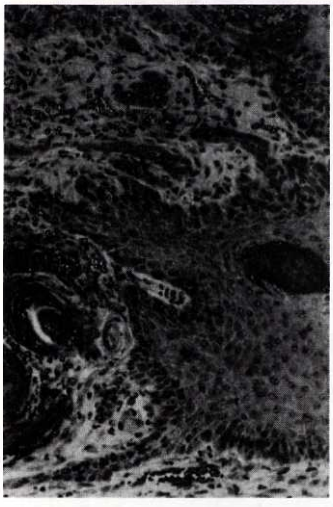

Fig. 2. Well-differentiated keratinizing squamous cell carcinoma of lip in Group III rat. H-E, ×96.

Tumor incidence

The incidence, location, and histologic type of tumors is given in Table 3. One squamous cell carcinoma of the lower lip was found in Group I and two in Group IV (Fig. 2). They were all growing in close connection to the entrance of, but not in, the test canal. Squamous cell carcinomas of the oral cavity, located in the crevicular epithelium close to the orifice of the lip canal were found in two rats of Group V and in one rat of Groups II and IV, respectively (Fig. 3). Squamous cell carcinomas of the ear duct were found in one rat in Groups I and III, respectively. One nasal squamous cell carcinoma was found in a rat of Group II and one forestomach tumor was found in a Group V rat. None of those squamous cell carcinomas had metastasized and no animals had more than one malignant tumor. The incidence of squamous cell carcinomas of the head and neck region did not significantly differ between the different groups. However, the total number of tumors was significantly higher in Group III as compared to Groups I, II and VI (Table 3), ($P < 0.05$). The difference between Groups IV and V in tumor incidence was not statistically significant.

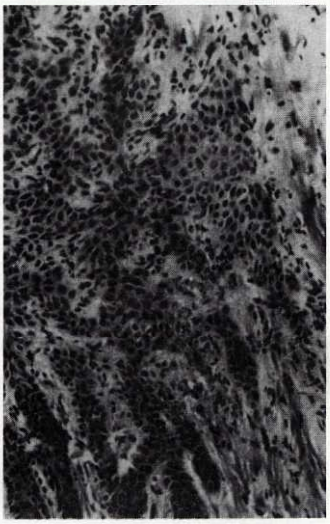

Fig. 3. Poorly differentiated squamous cell carcinoma of oral cavity in Group I rat. H-E, ×130.

Fig. 4. Spindle cell sarcoma of stomach in Group III rat. H-E, ×196.

Table 3. Incidence, location, and histologic type of tumors in the different groups (numbers of rats affected).

Group number	I	II	III	IV	V	VI
Effective number of rats	12	13	15	12	12	8
Type of lesion and location						
Squamous cell carcinoma						
Ear duct	1		1			
Lip	1			2		
Oral cavity		1		1	2	
Nose		1				
Forestomach					1	
Squamous cell papilloma						
Forestomach				1		
Lip					1	
Adenocarcinoma						
Breast			2			
Colon		1				
Hepatoma						
Liver					2	
Pheochromocytoma						
Adrenal gland			1	1	3	
Carcinoid						
Lung				1		
Sarcoma						
Stomach			1			
Salivary gland			1			
Scrotum			1			
Leukemia					1	1
Adenoma						
Breast	1		2	1		
Adrenal cortex			2			
Cavernous hemangioma						
Gingival mucosa			1	1	2	
Fibrous histiocytoma						
Breast			1			
Desmoplastic fibroma						
Skin		1				
Total	3	4	13	8	12	1
Number of tumor bearing animals	3	3	8	5	7	1
Number of malignant tumors	2	3	7	5	9	1

Preneoplastic lesions and reactive changes

Dysplasia of squamous epithelium of the lip was most commonly seen in Group V. (Table 4).

Hyperplastic lesions were most prevalent in the rats of Group III. Hyperplastic lesions of the labial epithelium was not only confined to the test canal. One striking observation was the presence of foreign body giant cell granulomas in the connective tissue of the lips which were significantly more common in Group V rats (Table 4). These granulomas were not only more prevalent, but their size was also significantly larger in Group V than in the other groups.

The kidneys were enlarged with variable degrees of rat nephrosis in most of the animals. The average grade of rat nephrosis in the different groups is shown in Table 5. The results clearly show that the Lewis rats were not affected by severe rat nephrosis but mostly by mild forms.

Discussion

There were no significant differences in survival time between rats treated with snuff or virus alone, compared to rats treated with a combination of snuff and HSV-1 (Table 2). Neither was there significant difference in survival between 4-NQO treated rats and rats treated with 4-NQO + snuff. The 6-month longer survival time of the Lewis rats as compared to the Sprague-Dawley rats may be explained by the fact that these rats had much less pronounced rat nephrosis than the Sprague-Dawley rats (Table 5). The rat-nephrosis is related to a high diet protien intake, and is known to be one of the most important factors causing early health detoriation and death in ageing laboratory rats, especially male Sprague-Dawley rats (21). It is unlikely that treatment differences could account for the longer life span of the Lewis rats.

The weight gain followed the same course for both Lewis and Sprague – Dawley rats during the experiment, but Groups II, III, and V had a slower weight gain than Groups I, IV, and VI (Fig. 1). There are several possible explanations to the slower weight gain of the snuff treated groups. One could be that the snuff exposure resulted in general toxic effects. Another is an increased metabolic activity induced by nicotin. A third possible explanation is the fact that snuff administration resulted in chronic inflammaton of the lip region with soreness and pain which might have prevented maximum food intake.

There were no significant differences

Table 4. Incidence of reactive and preneoplastic lesions in the different groups (numbers of rats affected).

Group number	I	II	III	IV	V	VI
Effective number of rats	12	13	15	12	12	8
Type of lesion and location						
Giant cell granulomas						
Lip (small)	1	2	4			1
(large)	1	3	1	2	10	1
Hyperplasia						
Lip (minor)	4	8	12	5	5	1
(severe)	1	3	2		3	
Crevicular epith. (minor)	5	5	8	4	3	1
(severe)		1	2	1	3	
Forestomach (minor)		1	3	4		
(Severe)		1	2		1	
Dysplasia						
Lip					2	
Crevicular epithelium			2	1	2	
Forestomach					1	

Table 5. Incidence and severity of rat nephrosis in different groups.

Group number	I	II	III	IV	V	VI
Effective number of rats	12	13	15	12	12	8
No nephrosis	1	1	1	5	1	
Grade 1 (+)	3	5	6	7	10	
Grade 2 (++)	2	3	4		1	5
Grade 3 (+++)	6	4	4			3
Average of grade of severity of nephrosis	2.1	1.8	1.7	0.5	1.0	2.7

in the incidence of tumors in specific organs or of specific histologic types between the different groups but there was a marked and statistically significant difference between the total number of tumors in Group III versus Groups I, II, and VI. The difference in total tumor incidence between Groups IV and V was not statistically significant. Although there are no earlier reports on differences in spontaneous or induced tumor incidence between Lewis and Sprague-Dawley rats, we regard it important to interpret the tumor incidence in Groups IV and V separately. The reason for using Lewis rats in the 4-NQO treated groups was the intention to explant the tumor cells into cultivating medium in order to examine for chromosomal abberations, for which inbred animals is a prerequisite. However, due to technical problems no cell growth was obtained.

The highest tumor incidence was found in Group III which had been subjected to both virus and snuff exposure. The results support that the combination of HSV-1 and snuff has tumorigenic properties and they are consistent with our earlier results and those of other investigators (9, 19). However, oral squamous cell carcinomas were not induced in this study after exposure to HSV-1 and snuff. This may be explained by differences in methods of infecting the rats. The absorbtion of the virus suspension in a cotton pellet before application in the test canal instead of direct application on the mucous membrane might explain the induction of fewer oral squamous cell carcinomas in this study. The intention when inoculating virus in the test canal was to study a possible local carcinogenic effect of the combination HSV-1 and snuff but our data do not support the presence of such local effects when using a cotton pellet as a vehicle for the virus suspension.

There may be several mechanisms to account for the higher tumor incidence in the group subjected to the combination of HSV-1 and snuff. One factor is the fact that chemicals in snuff inhibit the cytolytic properties of HSV-1 (18) which enables HSV-1 to express its possible carcinogenic properties. Other investigators have also shown that uv-inactivated HSV-1 may transform cells *in vitro* and that these cells when injected into the homologus host formed tumors which metastasize (17). Another possiblity is that some of the 2500 chemical substances in snuff (22) may act as initiators and chronic HSV-1 infection functions as a promoter, by inhibiting DNA repair by disruption of host DNA (23). Important chemicals in snuff are the tobacco specific N-nitrosamines (TSNA) which are present in mg/kg concentrations (22,24). Administration of these compounds result in tumors locally at the site of application as well as organ-specific tumors in nasal cavity, esophagus, lungs, and liver (24). The rats subjected to repeated HSV-1 infection exhibited signs of a generalized infection. The autopsies also confirmed this clinical observation in the two rats of Group I who died from encephalitis. It is possible that such a generalized HSV infection makes eucaryotic cells more susceptible to the carcinogens present in snuff, which may explain why the rats developed tumors distant from the site of HSV-1 inoculation and snuff application. A generalized HSV infection might also supress the immune response and if so facilitate tumor development. Another mechanistic possibility is that HSV-1 may, although it seems doubtful, even in its infectious state induce cancer and that snuff acts as a tumor promoter. The possibility that snuff acts only as a tumor promoter may be supported by the fact that the hyperplastic epithelial lesions induced by snuff are reversible (4). In the present investigation no significant increase in tumor incidence was found in group V (4-NQO + snuff) compared to group IV (4-NQO only). Thus we were not able to show that snuff functions as a tumor promoter in rats initiated with 4-NQO in the lip. Neither have we been able to promote cancer development with snuff in rats initiated with 4-NQO is the hard palate (Johansson *et al.*, to be published). Even though snuff appeared as a general tumor promoter in combination with HSV-1, it did not exert any specific promoting effects on the oral cavity. The reason for this is uncertain, but possibly polyphenols in snuff could function as local inhibitors of cancer development, as suggested earlier in a dose-response study in which rats, using the same experimental model were exposed to TSNA-enriched snuff. No correlation could be found between the TSNA content and tumor development which was ascribed to the presence of an inhibitor in the snuff (25, 8).

Of 13 rats who were exposed to HSV-1 and snuff, 6 developed malignant tumors compared with only 1 leukemia in the control group of 8 rats. No significant increase in the number of malignant tumors was found in the groups subjected to HSV-1 or snuff only. This has further underlined the hypothesis that HSV-1 and snuff interact in the development of malignant lesions. The relevant mechanisms are still unknown but further studies in this field are now conveyed.

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J. gen. Virol. (1989), **70**, 1773–1778. *Printed in Great Britain*

Key words: *HSV-1/acyclovir/tumour formation*

Effects of Acyclovir on Herpes Simplex Virus Type 1 Infection in Mice Treated with 12-*O*-Tetradecanoylphorbol 13-acetate

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(*Accepted 6 March 1989*)

SUMMARY

The purpose of this study was to determine whether infectious herpes simplex virus type 1 (HSV-1) has tumorigenic properties and, if so, whether inhibition of the cytolytic replicative cycle of the virus after infection enhances tumour development. Eighty mice were subjected to repeated inoculation of HSV-1 on their upper lips after scarification, and systemic administration of acyclovir (ACV). 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was used as the tumour promoter. The tumour incidence was compared to control groups each of 40 mice that were either not treated with ACV, not treated with TPA, not infected with HSV or only scarified. In the virus-infected group treated with ACV and TPA, 25% of the animals developed tumours. In the HSV-infected group treated with TPA only, 25% of the animals also developed tumours. The uninfected animals which were not treated with TPA developed tumours to a significantly lesser degree. In conclusion, the combined effects of HSV-1 and TPA, with or without ACV treatment, resulted in a significant increase in the number of tumours in comparison to the control groups.

INTRODUCTION

The role of herpes simplex virus (HSV) in human cancer has been the subject of great interest and controversy. It has clearly been established that inactivated HSV-1 is capable of transforming cells *in vitro* (Rapp, 1980) and circumstantial evidence supports the conclusion that HSV is associated with some human cancers (Shillitoe & Silverman, 1979).

Burns & Murray (1981) reported that inoculation of HSV-2 on abraded mouse lips followed by u.v. irradiation and exposure to 12-*O*-tetradecanoylphorbol 13-acetate (TPA) application resulted in the development of both squamous cell carcinomas and papillomas of the lip. They concluded that the inactivation of HSV by u.v. irradiation after infection allowed the virus to express its inherent oncogenic capacity when combined with the tumour promoter. Repeated exposure or persistent infection by HSV has previously been suggested as a possible mechanism for oncogenic transformation but reports confirming this are lacking. However, long term administration of snuff in combination with repeated HSV infections results in the development of squamous cell carcinomas both in the oral cavity of rats (Hirsch *et al.*, 1984*a*) and in the cheek pouch of Syrian hamsters (Park *et al.*, 1985). The carcinogenic effects of the combination of tobacco and HSV have been explained by the inhibitory capacity of tobacco extracts on cytolytic HSV infections (Hirsch *et al.*, 1984*b*). The tumorigenic effect of the combination of HSV and tobacco is not localized at the site of application as tumours are also found at distant sites in rats exposed to snuff after HSV infection (P.-A. Larsson *et al.*, unpublished results).

Acyclovir (ACV) is a specific antiviral drug that blocks viral replication by acting as a terminator of DNA elongation. Long term bioassay studies of ACV have not demonstrated any

carcinogenic properties in rodents, nor has it shown any transforming effects in eukaryotic cells (Tucker, 1982).

The present study was designed to determine whether exposure of mouse lips to infectious HSV-1, with or without the administration of a tumour promoter, would result in tumour development and whether inhibition of the cytolytic replicative cycle of the virus would then enhance tumour incidence. Systemic ACV treatment was used for inhibition of HSV-1 replication and local application of TPA was used for tumour promotion.

METHODS

Mice. Seven-week-old female and male mice (Swiss Albino, own breed) were kept in plastic cages, in 12 groups of 20. Males and females were kept separately. The animals were fed a standard pelleted diet (Astra Ewos) and tap water *ad libitum*. Temperatures in the animal quarters were kept at 22 ± 2 °C and the light/dark cycle was 12 h.

Chemicals. ACV was kindly supplied by Burroughs-Wellcome and TPA (1·425 mg/ml) was purchased from Sigma.

Virus. HSV-1 strain F ($1{\cdot}3 \times 10^7$ p.f.u./ml) was kindly supplied by B. Roizman. The techniques for preparation of virus stock suspensions and for the plaque assay of virus in GMK cells have been described in detail elsewhere (Vahlne *et al.*, 1981).

Experimental design. This is summarized in Table 1. The experiment started when the animals were 7 weeks old and their initial weight was, on average, 20 g. The 240 animals were divided into four groups (II to V) of 40, with 20 of each sex in each group, and one group (I) of 80 mice, 40 males and 40 females. All animals were scarified on both sides of the upper lip once a month. In groups I, II and III, virus suspension was thereafter applied to the scarified area of the lip; the animals in groups IV and V were mock-infected. The virus suspension was applied topically in a total volume of 25 µl at each application. Two of the virus-exposed groups (I and III) and one of the mock-infected groups (IV) received 0·5 mg ACV by intraperitoneal injection, at days 2, 4 and 6 after each inoculation. Two of the virus-infected groups (I and II) and one of the mock-infected groups (IV) were also exposed to TPA which was applied every 2nd, 4th and 6th day during weeks 2, 3 and 4 of each month. Ten µl of the drug was applied topically to the lip each time. TPA treatment was continued for 13 months in total. All mice were observed every other day until the experiment was terminated after 15 months when the mice were sacrificed by ether anaesthesia.

Morphological methods. The mice were killed at the termination of the study and underwent a complete post-mortem examination. Microscopic examination was performed on the lip, heart, lung, forestomach, liver, urinary bladder, kidneys, spleen and grossly abnormal organs or tissue. The specimens for this were fixed in 4% buffered formalin solution and embedded in paraffin. Sections (5 µm) were stained with haematoxylin and eosin according to the Weigert–van Gieson method.

Serological analyses. At the end of the experiment 1 ml of blood was drawn by cardiac aspiration from each animal for the determination of antibodies against HSV-1. The titres were established by an ELISA-based technique as described previously (Svennerholm *et al.*, 1984). Titres less than 1/100 were regarded as negative.

Statistics. The tumour incidence was calculated in each experimental group and factor analysis was done for the studied chemical compounds, as well as the combinations of these. Statistical significance of the results was calculated with Fisher's exact test (two-tailed). $P \leqslant 0{\cdot}05$ was regarded as statistically significant.

RESULTS

Mice which had been inoculated with virus without subsequent ACV treatment developed typical vesicular–ulcerative lesions within 3 days. Seven to 10 days after inoculation the lesions were encrusted and proceeded to the state of complete healing. The mice inoculated with virus

Table 1. *Experimental design, showing the monthly treatment of animals during 13 months of the 15 month experimental period*

	Week	1	1	1	1	2	3	4
	Day	1	2	4	6	(2,4,6)	(2,4,6)	(2,4,6)
Group								
I (80)*	Scarified + HSV-1	ACV	ACV	ACV	TPA	TPA	TPA
II (40)	Scarified + HSV-1	–	–	–	TPA	TPA	TPA
III (40)	Scarified + HSV-1	ACV	ACV	ACV	–	–	–
IV (40)	Scarified	ACV	ACV	ACV	TPA	TPA	TPA
V (40)	Scarified	–	–	–	–	–	–

* Number of mice in each group is shown in parentheses.

Table 2. *Total distribution of tumours found in the different groups*

Organ	I (n = 52)	II (n = 23)	III (n = 28)	IV (n = 13)	V (n = 24)
Lip	6 (0)*	2 (0)	0	0	0
Skin	2 (2)	1 (0)	0	0	1 (0)
Lung	0	1 (1)	0	0	1 (1)
Kidney	1 (1)	0	0	0	0
Breast	4 (3)	2 (2)	1 (1)	0	1 (1)
Total	13 (6)	6 (3)	1 (1)	0	3 (2)
Animals with tumours (%)	25	25	4	0	12

* Numbers of malignant tumours are shown within brackets.

and treated with ACV developed simple superficial wounds on their lips, as did those which were only scarified. These wounds healed within 4 days.

Owing to extensive cannibalism we were able to perform autopsies on only 140 of the 240 mice, which is therefore the effective number of animals in the study.

The type and location of all tumours found are presented in Table 2. Tumours of skin, lips and breast were all obvious at gross inspection. All eight lip tumours in groups I (six) and II (two) were squamous cell papillomas characterized by epithelial proliferation with hyper- and parakeratosis (Fig. 1). The squamous epithelial cells varied from mature to moderately dysplastic (Fig. 2). Evidence of invasion was not present. The two skin tumours found in group I were both early squamous cell carcinomas (Fig. 3) with one of these localized on the cheek close to the lower lip. The skin tumour in group II was a tail squamous cell papilloma with slight dysplasia. The lung tumours found in groups II and V were both in the range of moderately to well differentiated papillary adenocarcinomas. The two kidney tumours in groups I and V were both renal cell tumours composed of clear and granular cells. The four breast tumours in group I were moderately differentiated invasive adenocarcinomas. In group II one breast tumour was an adenocarcinoma similar to those in group I and the other was a squamous cell carcinoma. The breast tumours in groups III and V were both relatively poorly differentiated adenocarcinomas.

Histological examination of the lip specimens also revealed that the lips of almost all the animals in groups I, II and IV had focal hyperplasia of squamous epithelium with pronounced hyperkeratosis (Fig. 4). All groups had a moderate inflammatory reaction and a pronounced fibrosis in the subepithelial layers of their lips without any obvious differences between the five groups.

Serological analysis of the animals for antibodies against HSV-1 revealed positive titres in all mice of groups I, II and III, whereas for all animals of groups IV and V analysis revealed negative titres. There was no significant difference in antibody titres between the animals of groups I, II and III.

DISCUSSION

The present study has shown that repeated HSV-1 infection in the lips of mice treated with ACV followed by TPA administration for up to 13 months results in squamous cell tumours in the treated area. However mice subjected to HSV-1 infection and TPA treatment only also developed squamous cell lip tumours. Animals which were not treated with the combination of HSV-1 and TPA developed no lip tumours. The difference in tumour incidence between groups I and II was not significant. However, if the results for the animals that received HSV inocula and TPA, with or without acyclovir (groups I and II), are added together, the differences in incidence of lip tumours as well as of all tumours are statistically significant ($P \leqslant 0{\cdot}05$) when compared with animals not receiving this combined treatment (groups III, IV and V).

Burns & Murray (1981) used a classical model of initiator, cofactor and promoter to study the incidence of tumours in the treated area, i.e. the mouse lip. When HSV-2 infection was followed by u.v. irradiation for inactivation of virus infectivity, a significant number of lip cancers was found. The latent period for development of tumours after virus inoculation and u.v. irradiation

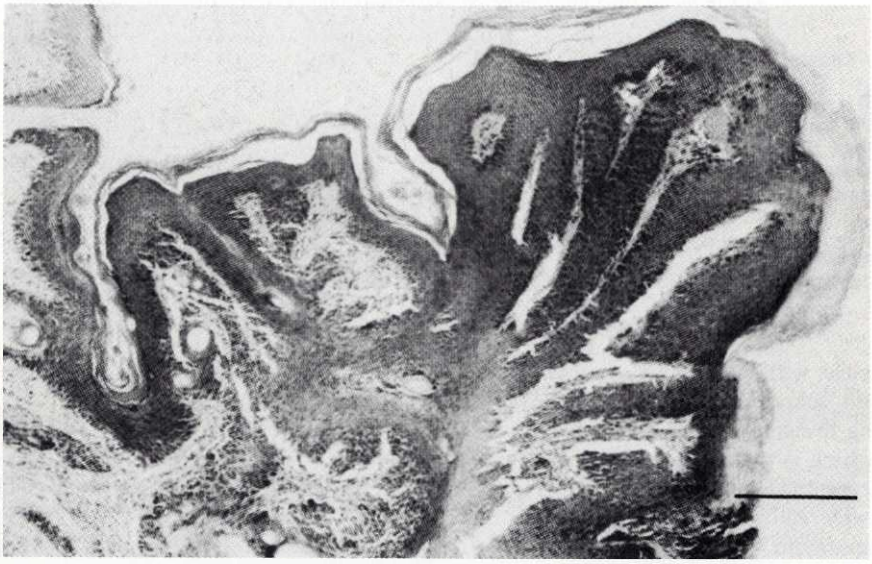

Fig. 1. Squamous cell papilloma of the upper lip in a mouse treated with HSV-1 followed by ACV and TPA (group I). Bar marker represents 0·5 mm.

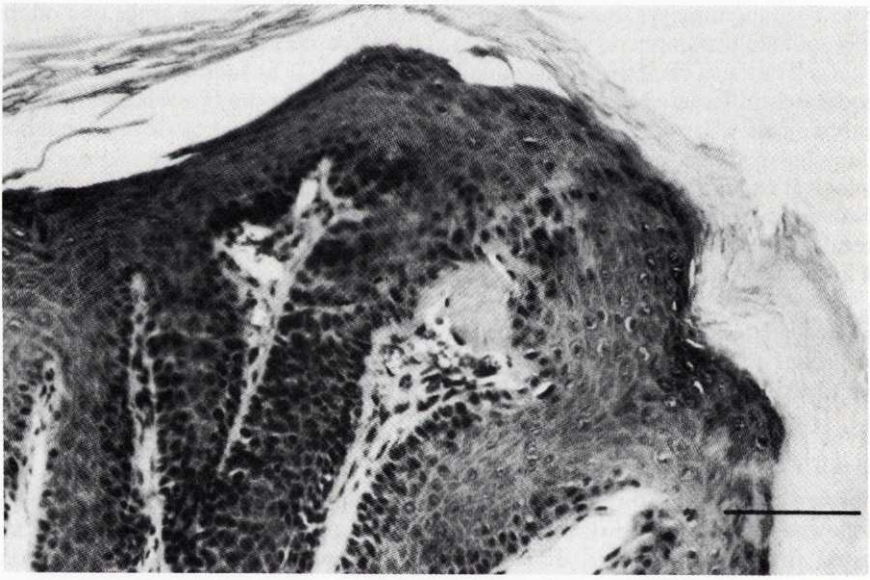

Fig. 2. Higher magnification of Fig. 1. Note the mildly dysplastic epithelium with hyper- and parakeratosis. Bar marker represents 100 μm.

varied between 10·8 and 24 weeks. In the present study, lip, skin and breast tumours developed after 11 to 14 months. Consequently, one problem in the present study was a high rate of loss of mice due to decreasing general health in the ageing animals and cannibalism. However, we considered the long-term treatment to be important in studying the oncogenic properties of ACV-treated herpes infection in mice and therefore we accepted the high mortality among the animals. In the present study, TPA administration was discontinued after 13 months, 2 months

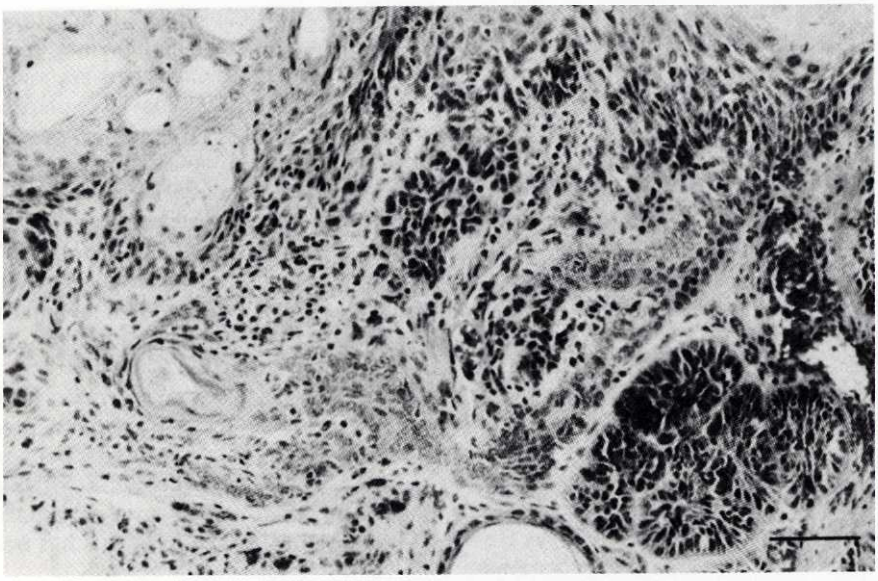

Fig. 3. Invasive squamous cell carcinoma of the skin in a group I mouse. Bar marker represents 100 μm.

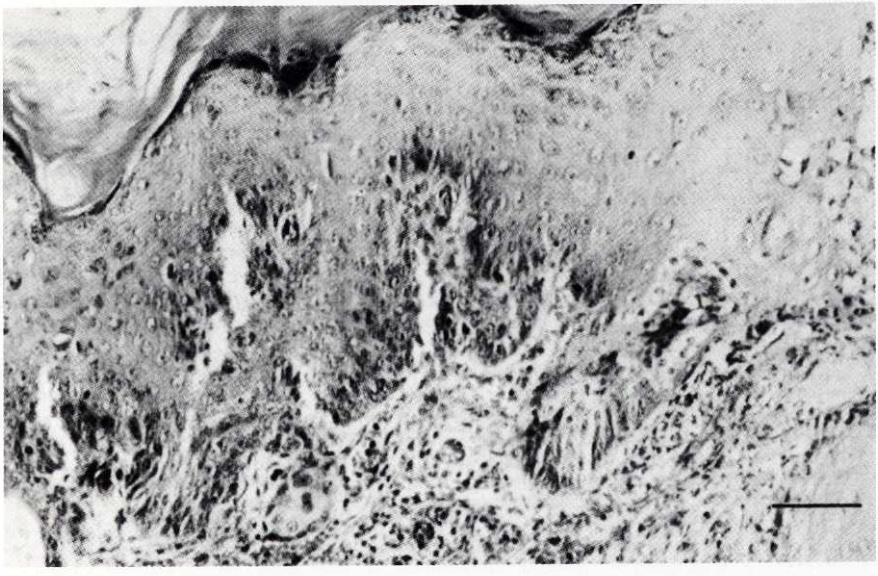

Fig. 4. Section of the lip in a group II mouse without a tumour. Note the marked epithelial hyperplasia and hyperkeratosis. Bar marker represents 100 μm.

before the termination of the study, which may have resulted in the disappearance of reversible lesions induced by TPA. On the other hand, with increased time of latency, irreversible and malignant lesions would have had a better chance to develop. The difference in tumour incidence between this study and the one by Burns & Murray (1981) might indicate that HSV-2 has a more potent initiating capacity than HSV-1. However, the different methods of inactivation of HSV after infection might well have been an important factor.

The present study was extended to the tumour response in the whole body, in addition to the lip area. The animals in groups I and II developed more tumours in the lung, kidney and breast compared to the animals in groups III, IV and V which, although the numbers are low, might suggest that the tumour formation after treatment with HSV-1 and TPA may take place at sites other than the site of application. We have also found that in rats, intra-orally exposed to the combination of HSV-1 and tobacco, tumours distant to the oral cavity are induced (P.-A. Larsson *et al.*, unpublished results). Most cells in mice are permissive to HSV infection, and in mice inoculated orally with HSV the virus will probably spread systemically. Also, locally applied chemicals are absorbed and generally spread within the body. Thus, both HSV and TPA, as well as systemically administered ACV, might exert their effects on different cells distal to the site of application. Another explanation could be that a generalized HSV infection might depress the immune response of the animals, thereby facilitating spontaneous tumour development.

In this study we were not able to show that blocking of the lytic replicative cycle of HSV-1 by ACV results in an increased tumour incidence, even when followed by tumour promoter treatment. In fact the difference in tumour incidence outside the oral cavity between the different groups of mice might indicate a possible anti-tumorigenic effect of ACV, although the number of these tumours was too small to allow statistical evaluation. However our data are the first to provide evidence that infectious HSV-1 has tumorigenic properties. This has not been shown before and will require further study.

This study was supported by the Swedish Tobacco Company, the Swedish Dental Association and the Swedish Cancer Society.

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(*Received 28 October 1988*)

V

INHIBITION OF HERPES SIMPLEX VIRUS REPLICATION AND PROTEIN SYNTHESIS BY NON-SMOKED TOBACCO, TOBACCO ALKALOIDS, AND NITROSAMINES.[1]

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Running title: Tobacco, HSV replication, and protein synthesis.

1 This study was supported by grants from Göteborg Medical Society, Grant No. 89/167, Svenska Tobaks AB, Grant No. 8724, and Swedish Cancer Society, Grant No. 2555.

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3 The abbreviations used are: HSV, herpes simplex virus; HSV-1, herpes simplex virus type 1; DEN, dietyl-N-nitrosamine; TSNA, tobacco specific nitrosamines; NNN, N-nitrosonornicotine; NNK N-(N-methyl-N-nitrosamino)-1-(3-pyrridyl)butanone; GMK, green monkey kidney - cells; HEp-2, human epidermoid carcinoma No. 2 cell line; MEM, minimal essential medium; HPLC, high performance liquid chromatography; IdUrd, 5-iodo-2'-deoxyuridine; PBS, $Na_2H_2PO_4$ buffered NaCl 0.155 M, pH 7.4; MOI, multiplicity of infection; PFU, plaque forming units; phosphate buffered saline; ELISA, enzyme linked immuno sorbent assay; SDS, sodium dodecyl sulphate; TCA, trichloroacetic acid; PAGE, polyacrylamide gel electrophoresis; ICP, infected cell protein.

ABSTRACT

Both herpes simplex virus (HSV) and non-smoked tobacco have carcinogenic capacity, but the carcinogenicity of non smoked tobacco is weak. However, a prerequisite for HSV to cause cell transformation is that the virus induced cell lysis is prevented. In experimental animals, the combination of non smoked tobacco and HSV infection results in a higher incidence of tumors than snuff or HSV infection alone. In this study we have analyzed the inhibitory effects of snuff extract and certain tobacco chemicals, such as, nicotine, anabasine, dietyl-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. Snuff extract and nicotine induced in a significant reduction of HSV-1 attachment to cell membranes but anabasine DEN, NNN, and NNK did not affect adsorbtion 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 (τ-) ICPs 5, 11, 21, 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 τ- 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. In conclusion the study has shown that snuff extract induces a block at an early stage of the replicative cycle of HSV which results in an increased production of α-proteins in the infected cells and prolonged maintenance of cellular functions. This may be of importance for the HSV induced transformation and the development of HSV associated tumors.

INTRODUCTION

The oncogenic capacity of HSV has been thorroughly investigated. Both HSV-1 and HSV-2 can transform cells in vitro (1-3). 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 earlier been studied. Aqueos extract of snuff and condensated, smoked tobacco tar has been shown to dose dependently inhibit the replication of HSV-1 in in vitro cultured cells (4-6). Such substances which inhibit HSV replication and which 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 the developing of intraoral leukoplakias (7). Oral cancer has been attributed to the use of tobacco, i.e. both smoking and snuff dipping (8). Although, snuff by itself might induce tumors in the oral region, the carcinogenic effect is weak (9-11). However, the combination of HSV-1 and snuff has a pronounced carcinogenic effect in rats (12) and hamsters (13).

The block in HSV replication induced by both a non-smoked tobacco extract (4) as well as a smoked tobacco tar extract (6) is an early function i.e. before or at the level of DNA replication.

The aim of the present report is to further characterize 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.

MATERIALS AND METHODS

Cells.

GMK and HEp-2 cells were used in the experiments. The cells were grown as monolayers on 5 cm plastic petri dishes (NUNC Denmark) in Eagle's minimal essential medium (MEM) supplemented with 10 per cent fetal calf serum for the growing of GMK cells and with 8 per cent fetal calf serum forthe growing of HEp-2 cells. Sixty μg of bensylpenicillin and 100 μg of streptomycin were added per ml medium. The same media supplemented with antibiotics but only three per cent fetal calf serum were used for maintenance. The cells were grown in darkness and an atmosphere of 95 per cent air and 5 per cent 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 preparation of virus stock suspensions and for the plaquing of virus in GMK cells have earlier been described in detail (14).

Chemicals.

Preparation of snuff extract: Water extract was prepared from one brand of fresh Swedish snuff purchased on the open market in Sweden. Ten gram of snuff was mixed with 50 ml of MEM, and incubated at 37°C for one hour, centrifugalized twice at 1000 rpm for ten 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). The aqueous extract of snuff was analyzed as to the content of the tobacco alkaloids, nicotine, anabasine, and anatabine, - and the tobacco specific nitrosamines - NNN, and NNK (10). Water extracts were frozen at -40°C prior to analysis. The results of these analyses are shown in Table 1.

Tobacco chemicals: Nicotine, anabasine, and DEN were purchased from Sigma, St. Louis, Mo., USA. NNN and NNK were the kind gift from Dr. D. Hoffmann (American Health foundation New York). NNN and NNK were more than 99.5% pure according

to gas chromatography, HPLC, thin layer chromatography and mass spectrometry (15).

Radiochemicals: L-[^{35}S]-methionine with the specific activity of approximately 200 mCi/mmol was purchased from Amersham International, Amersham, England and [^{125}I]-IdUrd was purchased from New England Nuclear Corp., Cambridge, Mass., USA.

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 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 read 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, $1x10^6$ cells were seeded in 5cm Petri dishes, suspended in growth medium to which snuff extract was added to give the final dilutions 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 hours after which the cells were trypsinized and cell counts performed on a Coulter counter. Third, the effect of used drugs on cell protein synthesis was assessed by the dye-binding assay earlier described by Bradford (15). Cells suspended in growth medium with drugs added as described above were seeded on Petri dishes. After three days the cells were rinsed five times with PBS and scraped off with a rubber policeman. Cells were disolved in 0.5 ml PBS. This solution was ultrasonicated for six minutes and filtered through a Whatman gf/c filter. Dye-reagent (Bio-Rad, Richmond, California) was added and absorbance was read at 595 nm in a Philips PU 8625 spectrophotometer. Adsorbance values were compared to those obtained with control cultures with growth medium only.

Assay of HSV Attachment to Cells.

GMK cells were grown as confluent monolayers in a 96 well microtiter-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 with 0.1 M PBS and fixed in 0.02 % formaldehyde. Adsorbtion of virus was determined with an ELISA. After three washes in PBS-Tween 20 (0.05%), was added 100 μl of the HSV-1 mouse monoclonal antibody B1C1 (17, 18), diluted 1:100 in PBS-Tween 20, supplemented with 1 % bovine serum albumin. The plates were incubated for 60 min after which the washing procedure was repeated. To each well was added one hundred μl alkaline phosphatase conjugated Fab fragment goat anti mouse IgG (Jackson Immunoresearch Laboratories, West Grove, Pa., USA) at a dilution of 1:1000. Finally, After 60 min at 37°C, the plates were washed again and substrate, p-nitrophenyl phosphate (Sigma P 104, Sigma Chemical Company, St. Louis, Mo., USA) dissolved in 200 μl aquous diethanolamine solution, (Fluka Chemie AG, Buchs, Switzerland), was added. Adsorbtion was read repeatadly in a Biotek microplate reader at wave lengths 405 and 540 nm. Adsorbtion curves were then plotted versus 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 three ml of maintenance medium with as well as without the addition of test substances (Tables 4 and 5). The dishes were then frozen and thawn rapidly in three consecutive cycles, wherafter the cells and the medium was transferred to a centrifuge tube and centrifugalized for 10 min at 1000 rpm to remove cell debris. Plaque titration was performed to measure the progeny virus production (14). Five hundred μl of the supernatant, diluted from 10^{-1} to 10^{-6} was seeded on GMK cultures in duplicate. After 30 minutes the cultures were covered with plaquing medium containing 1 per cent methylcellulose, 2 per cent fetal calf serum, penicillin, and streptomycin of the same concentrations as in the maintenance medium. Plaques were counted after five 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 one h at room temperature whereafter the dishes were 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 produced 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 one hour of incubation at room temperature cultures were rinsed three times with Eagle's MEM and exposed to snuff extracts and chemicals at the concentrations stated above. For the labelling of immediate early proteins (α-proteins), cultures were incubated with [^{35}S]-methionine, approximately 25 μCi/culture, dissolved in methionine free Iscoves medium, (3ml/culture) containing snuff extracts or tobacco chemicals, from one to four hours post infection. To study the production of early proteins (ß-proteins), the infected cells were incubated for three hours in 5 per cent CO_2 at 37°C in maintenence medium with snuff extracts and tobacco chemical added as stated above. After this the cultures were rinsed and labelling was performed in the same way as for α-proteins from four to eight hours post infection. For labelling late proteins (τ-proteins), infected cultures were incubated for seven hours in 5% CO_2 at 37°C in maintenence medium with snuff extract and tobacco chemicals added and labeled from eight to twelve hours post infection as described above. At the end of the labeling period the cultures were rinsed three times with ice cold PBS, to terminate amino acid incorporation, harvested with a rubber policeman, dissolved in a small volume of PBS and centrifugalized for four min at 3000 rpm 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 per cent sodium dodecyl sulfate (SDS), 5'ß-mercaptoethanol, and 0.05 M Tris-hydroxychloride (pH 7.0). Fifty μl of the solubulized material from each culture was added to each well of the gel. In parallel, 50 μl of each sample was precipitated onto filter papers with 10% ice cold TCA. The precipitate was washed twice with 6% TCA, once with with ethanol/ether mixed in proportions 50:50 and once with ether only. The radioactivity of the TCA precipitates on dried filter papers was assessed by liquid scintillation.

Polyacrylamide gel electrophoresis was performed as described by Morse et al. (19), in a discontinous buffer system containing 0.1 per cent SDS. The stacking and

separation gel contained 3 and 9 per cent acrylamide respectively, cross linked with N,N'-diallyltartardiamide (2.6 per cent of acrylamide weight). All chemicals for gel preparation were purchased from Bio-Rad, Richmond, Ca., USA. Separation gel was 15 cm in length. Proteins used for molecular weight calibration were 14C-methylated-myosin, phosphorylase-b, bovine serum albumine, ovalbumin, carbonic anhydrase, and lysozyme (Amersham International, Amersham, England) 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 mw 100 000 and 92 500. Protein bands were designated according to Morse et al. (19). Adsorbance measurements of the autoradiographic images were performed in a Shimadzu CS 910 spectrophotometer equipped with a CR 1B chromatoscan. Amount of each protein was recorded and calculated as per cent of total adsorbance in lane, as well as per cent of TCA precipitable radioactivity added to the lane. The overall relationship of each individual protein between the variously treated cultures did not differ, no matter which of these methods was used.

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 measuring virus specific enzyme activity which directly reflects produced amounts of these enzymes. Cultures were infected with HSV at an MOI of 1 PFU/cell. Virus was adsorbed for one 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 maintenence medium containing snuff extract and tobacco chemicals as stated in Figs. 2a and 2b. After one, four, seven, and eleven hours respectively duplicate cultures were rinsed three times with ice-cold PBS, scraped off with a rubber policeman, dissolved in one ml PBS and frozen at -70°C. Enzyme assays for the determination of the DNA-polymerase and thymidine kinase activities were performed as described earlier (20, 21) on sample diluted 1:100. Assay time for DNA-polymerase was 180 min, and total amount of radioactivity available was 170,000 cpm.

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 (22).

RESULTS

Evaluation of Cell Toxicity.

Confluent GMK-cells cultures were exposed to snuff extract in dilution 1:1, 1:5, and 1:25 for six days. Light microscopic morfological changes, interpreted as signs of toxicity could be detected after 4 days in cultures exposed to undiluted snuff extract and after six days slight signs of toxicity were observed also in cultures exposed to snuff extract at dilution 1:5. However, no morfological signs of toxicity were detected in cells exposed to snuff extract at dilution 1:25. Clear morfological signs of toxicity were detected after three days in GMK cultures exposed to 1.0 mg/ml of nicotine, anabasine, and DEN. Slight toxic effects were also observed in cultures exposed 1.0 mg/ml of NNN and NNK after three days and clearly after six days, but no morfological signs of toxicity could be detected in cultures exposed to 0.1 mg/ml of the drugs used in these experiments.

Results of cell counts and quantitative analysis of soluble cellular proteins are presented in Tables 2 and 3. The data presented are the averages of three cultures. 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 obtained. The growth rate of GMK cells was affected by nicotin, anabasine, NNN, NNK, and DEN at the concentration of 1 mg /ml, and for NNN also at the concentration 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 in presence of snuff extracts, nicotine, anabasine, DEN, NNN and NNK were studied. After intervals ranging from 0 to 120 min, the amount of cell associated virus was determined. Data are presented in Fig. 1. Snuff extract diluted 1:2 induced a complete inhibition of attachment and also at dilution 1:5 attachment was affected. 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 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 an MOI of 1 PFU/cell, snuff extract diluted 1:5 reduced the HSV-1 production more than three log units (99.9 per cent), whereas snuff extract diluted 1:25 exerted a one log unit (90 %) reduction of progeny virus as compared to the controls. The reductions induced by snuff extract at dilution 1:5 and 1:25 were both statistically significant. The inhibiting effect on HSV replication of the snuff extract diluted 1:10 was pronounced at MOIs 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 the concentration 1.0 mg/ml, and DEN at 1.0 mg/ml also induced a significant reduction of HSV progeny. The effects on HSV production induced by nicotine, anabasine and DEN at lower concentrations were not statistically significant, nor 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 obtained, see Figs. 2, 3a and 3b. At both labelling intervals one to four hours and four to eight hours post infection snuff extract induced an increase in the amount of ICP 4 in infected cells. This increase was most pronounced at the higher concentration of the tobacco extract. More of ICP 27 was also obtained by addition of snuff extract to the cultures but the increase in quantity of ICP 27 was initially more elevated by snuff at dilution 1:25 than 1:5. During the first two labelling intervals, also the relative amount of the early gene product ICP 6 was increased in the presence of the tobacco extract (Figs. 2, and Fig. 3a and b). Nicotine at the relatively toxic concentration 0.5 mg/ml gave similar effects on the amounts of ICPs 4, 27, and 6 during the first

labelling period. At later labeling periods, nicotine at this concentration gave a general decrease of all HSV protein produced.

The production of τ-proteins, measured at eight to 12 hours post infection, was reduced by addition of snuff extract at dilution 1:5 to HSV infected cultures. The relative production of ICP 29 in cultures exposed to snuff at dilution 1:5 was reduced compared to the production of these proteins in control cultures. However, the production of ICP 29 in cultures exposed to snuff at dilution 1:25 was somewhat increased compared to the controls (Fig. 3c). The production of ICPs 5, 11 (gB) and 21 (gE) was reduced by snuff extract at both dilutions tested. The production of τ-proteins increased during the course of infection in control cultures but in cultures exposed to snuff the increase in production of τ-proteins was markedly reduced. The relative reduction in τ-protein production in snuff exposed cultures was still seen when followed up to 24 hours post infection.

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 ß-proteins. The activity of these viral enzymes versus the course of infection is plotted in Fig 4. The activity of thymidine kinase increased in control cultures from one to eleven h post infection. Eleven hours post infection snuff extract at dilution 1:5 caused a 88 per cent decrease in activity of thymidine kinase, and in the presence of snuff extract at dilution 1:25 a reduction of 72 per cent was obtained (Fig. 4a). The activity of DNA polymerase increased from one to eleven hours post infection but to a less degree 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 per cent by snuff extract at dilution 1:5 and by 77 per cent by snuff extract at dilution 1:25 (Fig. 4b).

Nicotine and anabasine at the concentration 1.0 mg/ml exerted a 23 per cent and 39 per cent depressive effect on thymidine kinase activity and depressed the activity of DNA polymerase by 34 per cent and 54 per cent, respectively, as measured 11 hours post infection. At lower, non toxic concentrations of these drugs no effects on activity

of thymidine kinase and DNA polymerase were obtained. DEN, NNN and NNK had no effects on thymidine kinase or DNA polymerase at the concentrations tested.

Production of Actin in HSV Infected Cells.

The autoradiographic images of the SDS-PAGEs revealed that the decrease in production of the cellular protein actin four hours post infection and later in HSV infected cells was significantly affected by the addition of snuff extracts to the maintenance medium (Figs. 2 and 5). From four to eight hours post infection, the relative synthesis of actin was 55 per cent higher in cultures exposed to snuff extract at a dilution of 1:5 than that in control cultures. In cultures exposed to snuff extracts at dilution 1:25, the actin production from four to eight hours was slightly but not significantly higher than in control cultures. After eight h the production of actin almost disappeared in control cultures, but still contributed to 7.5 per cent of the total protein synthesis in cultures exposed to snuff extract in dilution 1:5 and 2.2 per cent at dilution 1:25. No significant effect on actin production in HSV infected cultures were seen when non toxic doses of nicotine, anabasine, DEN, NNN, or NNK were added.

DISCUSSION

Aqueous extract of snuff has earlier been shown to dose dependently inhibit HSV replication (4). This was also confirmed by the present study. The inhibitory effect of snuff extract on HSV attachment to cells (4) could not be attributed to any of the tobacco chemicals studied. However, in all experiments on HSV replication and virus protein synthesis, snuff extract or tobacco chemicals were added one hour post infection in order not to interfere with the HSV adsorption to the cells.

Although the more extensive investgation of cell toxicity in this study revealed that snuff extract, even at the dilution 1:5, exerted a toxic effect on 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 (4-6) probably is a specific interaction between viral replication and tobacco chemicals.

Nicotine and anabasine are the water soluble alkaloids that are 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 have been shown to be present in the saliva of snuff dippers (23, 24). 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 was far below the levels at which they are toxic to cells or interfere with the viral replication. DEN is another nitrosamine which is present in significant amounts in American snuff, but only occasionally in Swedish snuff (25). DEN has earlier been shown to inhibit HSV replication in vitro at a concentration of 1.0 mg/ml (26). The rationale for studying DEN was to compare its effect on HSV replication to those possibly obtained with 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 found to be 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 the concentration of 0.5 mg/ml was significant but at the concentration 0.1 mg/ml no inhibitory effect was obtained. Anabasine, an alkaloid related to nicotine and present in significant amounts in tobacco products, was also shown to inhibit viral replication. However, this was found 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 HSV functions and is not secondary phenomenon due to cell toxic effects. Furthermore, at low MOIs the nitroseamines NNN, and NNK, as well as anabasine, and nicotine might have an effect on HSV replication not seen at the MOI (one PFU/cell) studied. Neither can a possible synnergistic effect of the tobacco chemicals be excluded.

The tobacco induced inhibition of HSV replication has been reported to appear at an early stage in the virus' replication cycle, i.e. before or at the level of DNA replication (4-6). This is supported by the effects of snuff extract on HSV protein synthesis in the infected cells presented here. 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-,ß1-, ß2, τ1-, and τ2-proteins, of which α- and ß-proteins are synthesized prior to progeny HSV DNA (27-29). Synthezis of α-protein requires no prior protein synthesis but ß-proteins require previous synthesis of α-proteins and in turn τ-proteins require previous ß-protein synthesis for their production. An inhibition of ß- and τ-protein synthesis also implies a prolonged production of α-proteins (27, 30). The increased production or accumulation of α-proteins and the decreased production of τ-proteins, in the presence of tobacco extracts, implies 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 temporally located before synthesis of $ß_2$-proteins but after $ß_1$-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 as such. Stich et al. (5) and Oh et al. (6) found no effect on the synthesis of α- or ß-proteins when HSV infected vero cells were exposed to snuff extracts or smoked tobacco tar, but found a significant reduction of τ-protein synthesis. They concluded that the reduction of τ-protein synthesis was secondary to the supression of the DNA-synthesis. The results of the present study disprove that tobacco chemicals acts solely at the level of the DNA synthesis in HSV replication.

Another possible mechanism is that snuff extract interferes with the virion host shut-off function. Both the finding of prolonged synthesis of ICP 4 and ICP 27 and of cellular actin in the infected cells exposed to snuff extract support this. The HSV host shut-off

can be divided into two phases, one primary, mediated by a virion component which does not require the synthesis of viral DNA and one late phase reducing the remaining levels of host protein synthesis (31-33). The latter requires the expression of viral genes. Both phases are reported to be coded for by the same gene, mapping between 0.602 and 0.606 encoding for a 58 kilodalton protein. The finding that snuff extract administration induces a prolonged synthesis of cellular actin in HSV infected cells also indicates that tobacco chemicals might help cells survive an herpetic infection despite the toxic effects of the tobacco chemicals. This may be of importance for the carcinogenic effects of the combination of tobacco and HSV observed in laboratory animals (12, 13, 34).

A third possible mechanism could be that substances in snuff extract interacts 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 thefunctional and physical properties of these have been extensively investigated (35, 36, 37). The α-proteins have been shown to act in trans to affect the transcription of specific subsets of viral genes. ß-protein synthesis is dependent only on the expression of functional α-proteins, whereas the production of τ-proteins also requires various degrees of viral DNA synthesis for their production (14). After the 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 (38, 39). The mechanism of transport of ICP 4 from the cytoplasm to the nucleus and localization in the nucleus is not known. A possible effect of the substances in tobacco could be an interference with ICP 4 which - inter alia - would lead to a failiure in the autoregulative mechanisms of the ICP 4 synthesis (35).

ICP 4 and ICP 27 are potent trans-acting transcriptional activators (40). ICP 4 is also a potent activator of HIV replication, a potent amplifier of the rabbit betaglobulin gene, and bovine papilloma virus DNA in human cells and thus it has an effect also on alien DNA (41-43). 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. submitted).

The results of the present study 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 study.

ACKNOWLEDGMENTS

We are grateful to Dr. Klaus Brunnemann, American Health Foundation, New York and Dr B-G Österdahl, Swedish Food Administration, Uppsala, Sweden, for performing the analyses of tobacco specific nitrosamines in snuff extract and to Dr. D. Hoffmann American Health Foundation, New York, for providing NNN and NNK.

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Table 1. Analytical profile of the Swedish snuff extract used in the experiments with regard to tobacco alkaloids and tobacco specific nitroseamines (μg/ml).

SAMPLE	pH	NICOTINE	ANABASINE	NNN	NNK
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

Table 2. Effect of snuff extracts on cellular protein production and cell growth in green monkey kidney cells.
* Denotes significant difference as compared to control.

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

Table 3. Effects of tobacco chemicals on cellular protein production and cell growth on GMK cells. * Denotes significant difference compared to control.

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

Table 4. Effect of snuff extract at different concentrations on HSV production and at different MOIs. * Denotes significant difference compared to control.

Dilution of snuff extract	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

Table 5. Effect of tobacco chemicals on HSV-1 replication. Cultures were inoculated at an MOI of 1 PFU/cell. * Denotes significant difference compared to controls.

Substance	concentration mg / ml	Produced HSV/culture (log p.f.u.) 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	

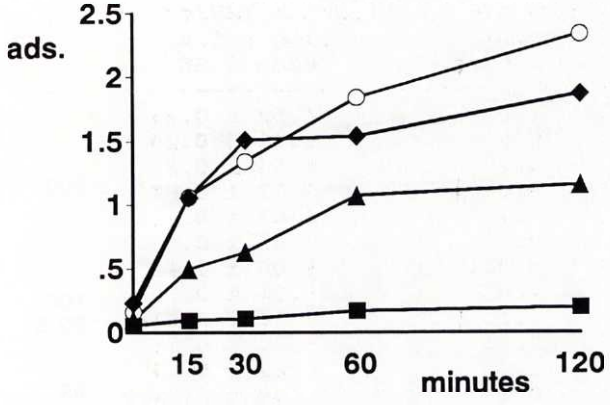


Figure 1: Attachment of HSV to GMK cells in the presence of snuff extract and nicotine. The amount of cell associated virus, as measured by adsorbance, is plotted versus the time of attachment (mean; n=12). SE varied between 0.01 and 0.13. Eagle's medium only (empty circles), nicotine 1 mg/ml (filled diamonds), snuff 1:5 (filled triangles), and snuff 1:2 (filled squares).

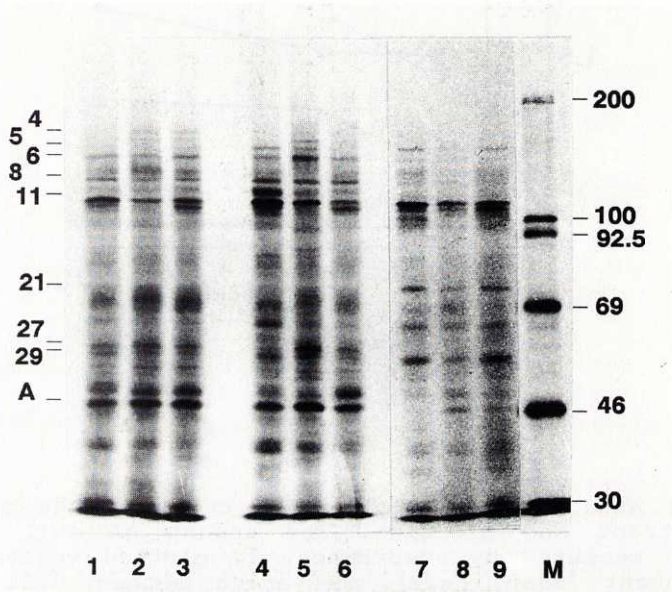


Figure 2: The autoradiographic immage of HSV-1 proteins electrophoretically separated in a 9.25 % polyacrylamide gel. Proteins from infected cells labelled with ^{35}S-methionine 1-4 hours post infection (lane 1-3), 4-8 h post infection (lane 4-6) and 8-12 h post infection (lane 7-9). Molecular weight 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 at left indicate the different ICPs according to Morse et al. (23).

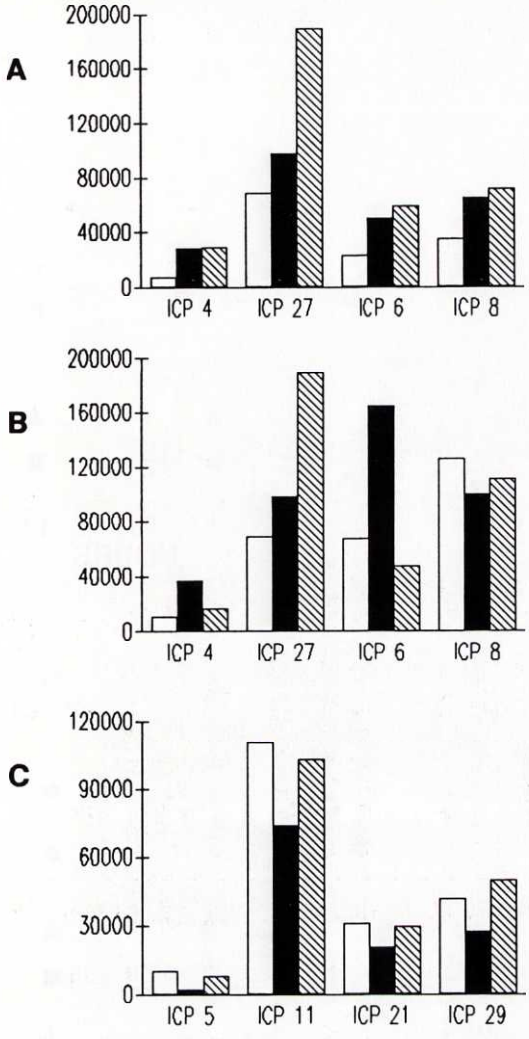


Figure 3: A: Synthesis of the immediate early and early HSV proteins ICPs 4, 27, 6 and 8, 1-4 h post infection in cultures exposed to snuff extract at dilution 1:5 and 1:25 and controls which were not exposed to Eagle's MEM only. B: Synthesis of ICPs 4, 27, 6 and 8, 4-8 h post infection in cultures exposed 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 (filled bars), snuff diluted 1:25 (hatched bars), and controls (empty bars). Values indicate the densitometric adsorbance obtained in the autoradiographic immages.

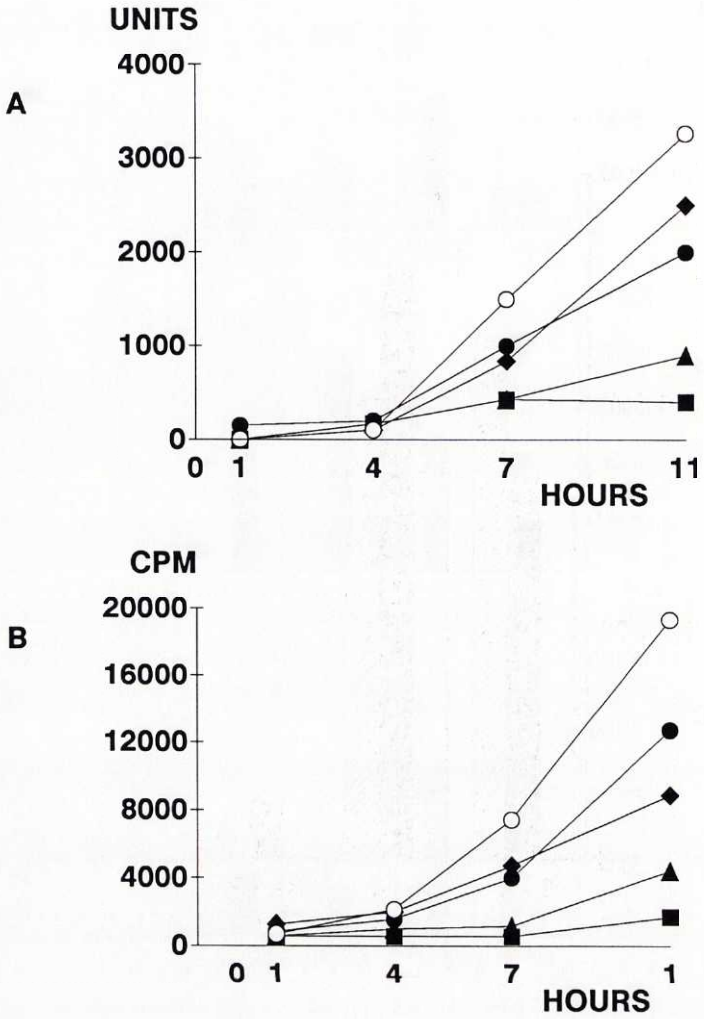
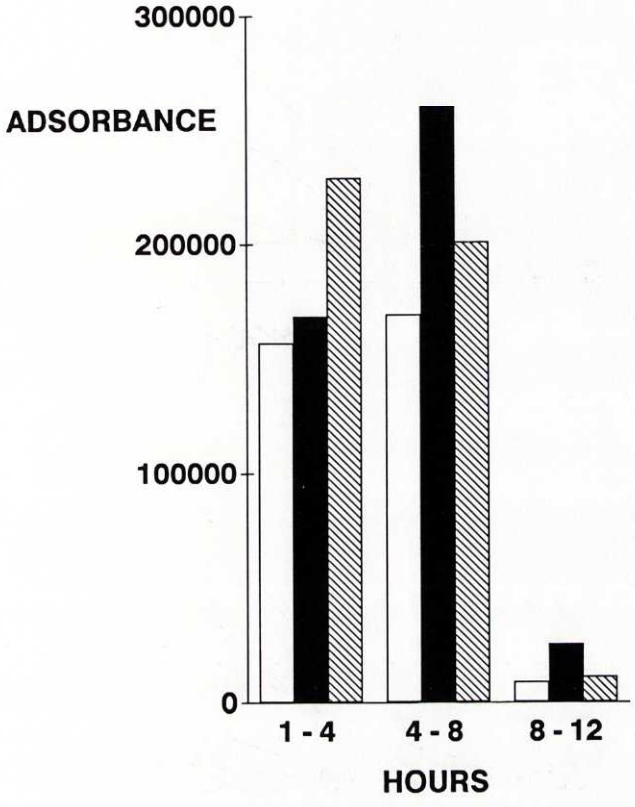


Figure 4: Production of viral thymidine kinase (A) and DNA-polymerase (B) measured as activity of these enzymes in HSV infected cultures 1-11 h post infection. Activity of thymidine kinase was calculated in units as described earlier (24) and DNA polymerase activity as counts per minute. Cultures were exposed to snuff extract at dilution 1:5 (filled squares) and 1:25 (filled triangles), nicotine 1.0 mg/ml (filled diamonds), anabasine 1.0 mg/ml (filled circles), and controls with Eagle's MEM only (empty circles).



Figre 5: Synthesis of cellular actin in HSV infected cultures during the course of infection in cultures exposed to snuff extracts diluted 1:5 (filled bars) and 1:25 (hatched bars), as well as, in controls exposed to Eagle's MEM only (empty bars). Synthesis was measured as adsorbance in the autoradiographic immages of the SDS-PAGEs.

VI

TOBACCO CONSUMPTION AND ANTIBODIES AGAINST HERPES SIMPLEX VIRUS IN PATIENTS WITH HEAD AND NECK CANCER[1].

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Running title: IgG antibodies to HSV in head and neck cancer.

1 This study was supported by grants from the Göteborg Medical Society Grant No. 89/169, Svenska Tobaks AB Grant No. 8724 and the Swedish Cancer Society Grant No. 2555.

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3 The abbreviations used in this paper are: HSV, herpes simplex virus; HSV-1, herpes simplex virus type 1; HSV-2 herpes simplex virus type 2; VZV, varicellae zoster virus; CMV cytomegalovirus; ICP, infected cell protein; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; ELISA, enzyme linked immuno sorbent assay; BSA, bovine serum albumin; FCS fetal calf serum; PBS, $Na_2H_2PO_4$ buffered NaCl pH 7.4.

ABSTRACT

Both herpes simplex virus (HSV) and tobacco have carcinogenic capacity. In this study we have analyzed whether a clinical relationship exists between HSV and carcinoma of the head and neck. Ninety patients with cancer in the head and neck region were investigated for tobacco consumption and IgG antibodies against HSV. Antibody titers were established with an enzyme linked immuno sorbent assay and antibodies against specific HSV-antigens were analyzed by Western blot. Seroreactivity was compared to an age matched control group of patients with arteriosclerotic disease in their lower limbs, a disease also closely related to extensive tobacco consumption. Prevalence of antibodies against HSV was around 90 per cent and did not differ significantly between cancer patients and controls, but antibody titers against HSV were significantly higher in the groups of cancer patients. The cancer patients also reacted more constantly (80 per cent) in Western blot against the early immediate protein ICP-4 compared to controls (50 per cent). This suggests a different course of an earlier herpetic infection in these patients with a prolonged exposure to early immediate HSV-proteins which may be related to an increased risk of developing head and neck cancer.

INTRODUCTION

Circumstantial evidence links herpes simplex virus (HSV) with cancer in man (1-5). Epidemiological studies have revealed a relationship particularly between the presence of antibodies to HSV-2 and cancer of the uterine cevix (5-7). It has been suggested that HSV-1 infection is associated with cancer development in the oral region. Shillitoe et al. (8) have reported that patients with untreated oral cancer have higher levels of IgM but not of IgG antibodies to HSV. The levels of anti HSV IgM in successfully treated oral cancers were similar to the levels in control subjects. Smith et al. (9) have reported significantly higher titers of anti HSV IgA in patients with untreated squamous cell carcinomas of the head and neck region, than in control subjects and healthy patients with successfully treated cancers of this region. An interaction between the use of tobacco products and HSV-1 infection in the development of cancer has been suggested (3, 10,11), as between smoking and HSV-2 infection for cancer of the uterine cervix (12).

The aim of this study was to identify whether a relationship exists between previous exposure to HSV, monitored as presence of IgG antibodies to HSV with ELISA and development of cancer in the head and neck. The control group was matched not only with regards to sex and age, but also for tobacco consumption. The rationale was that oral and laryngeal cancer predominantly affects male smoking individuals (13). Antibody responce to two other herpes viruses, CMV and VZV was assayed for comparison. As we have recently found that tobacco extracts block HSV replication at an early stage, giving an increase in immediate early infected cell proteins (Larsson et al., submitted), antibody responce to individual HSV proteins with special reference to the immediate early protein ICP 4 was also analyzed.

PATIENTS AND METHODS

Patients.

Ninety patients, 69 men and 21 women, with carcinomas of the head and neck region were studied. The average age was 65.5 ± 1.37 years. The cancer patients were all admitted to the Department of Otolaryngology, Sahlgren's Hospital, Göteborg, Sweden, during 1986. This clinic serves an urban area of 1.5 million inhabitants. The patients were divided into three groups according to the anatomic localisation of their tumors, patients with laryngeal cancer, (n=16), patients with oral cancer (n=28), and patients with cancers of other types and locations in the head and neck region (n=46). Analysis of age and sex distribution did not reveal any specific sex differences between the groups of cancers, except for laryngeal cancer which was found in only one woman during the period of study.

Controls.

The non-cancer control patients (58 men and 21 women) were patients treated at the Department of General Surgery, Östra Hospital, Göteborg. The rationale when chosing controls was to find a group of patients of matching age and sex distribution, and with similar smoking habits. They were volonteers, consecutively chosen among patients with lower limb ischemia and the only exclusion criterium was a history of malignant disease. The average age was 62.4 ± 1.62 years. The difference in mean age between cancer patients and controls was not statistically significant.

Histopathological Analysis.

Of the 90 patients with tumors 75 had squamous cell carcinomas and 15 non squamous cell carcinomas. One laryngeal cancer (n=16) was a non squamous carcinoma and 15 were squamous cell carcinomas of which four were low

differentiated, nine moderately and two highly differentiated. Of the oral cancers (n=28) two were non-squamous cell carcinomas, five were low, 18 moderately and three were highly differentiated squamous cell carcinomas. Twelve of the cancers, classified as other head and neck cancers, were non squamous cancers, located in the maxillary sinus (1), colli (2), ear duct (1), external ear (2), parotic gland (4), and thyroid gland (2).

Tobacco Habits.

The tobacco habits of cancer patients and controls were recorded by means of questionnaires. Of the cancer patients 78 per cent were or had earlier been tobacco users. In the control group 80 per cent of the patients were or had earlier been tobacco users. Three patients with different types of head and neck cancer and one control patient were snuff dippers, one patient with laryngeal cancer and four control patients were both snuff dippers and smokers. All other tobacco using cancer patients and controls were cigarette smokers. The tobacco habits in all groups of cancer patients as well as in controls are presented in Table 1. From this table it is evident that the most extensive tobacco habits were found in the control group while the group of unspecified head and neck cancer had the highest proportion of non-tobacco users. In the group of laryngeal cancer patients, all had a history of tobacco consumption. However, tobacco habits did not differ significantly between the groups except from the group of laryngeal cancers which was dominated by ex-smokers. These patients were older than the patients of the other groups which may to some extent explain this finding. Of the female control patients 80 per cent were smokers whereas 52 per cent of the female cancer patients were smokers. Of the male controls 93 per cent were smokers whereas in the cancer groups the proportion of smoking males was 86 per cent. No significant differences in tobacco habits could be found between men and women in the different groups of cancer patients, but in the total material significantly more women were non smokers and significantly more men were ex-smokers. The ratios of tobacco users versus non tobacco users did not differ between tumor patients and controls but the smoking habits of the control patients were heavier than in the groups of tumor patients.

Of the 169 patients, 4 per cent were both smokers and snuff dippers and 3.2 per cent were snuff dippers only. All of these were men. Four men with head and neck cancers were snuff dippers.

Patients and controls were classified into the groups of tobacco users and non tobacco users. Tobacco users were defined as having an actual or previous consumption of more than 2 cigarettes, one cigarr or 10 grams of snuff per day lasting at least one year.

Sera.

As soon as the cancer diagnosis was established and before treatment was initiated, 10 ml of venous blood was drawn for serological analysis in non heparinized vacutainer tubes. The blood was centrifugalized at 3000 rpm for ten minutes and serum was immidiately frozen at -30°C for later analysis. Sera from control patients were drawn and treated in the same way.

Enzyme Linked Immuno Sorbent Assay.

IgG titers against HSV, HSV-2, CMV, and VZV were established by an ELISA based technique described previously (14). Titers were expressed as the reciprocal of the dilution, giving an adsorbance higher than three SD over the mean of negative controls. The tests were performed on a 96 well plastic dish (Nunc no.269620, Denmark). One positive control, two negative controls, and ten test samples were analysed on each plate and all samples were analyzed parallelly on two different occasions. Titers less than 100 were regarded as negative. The antigen used for detection of antibodies against HSV is a sodium deoxycholate solubilized antigen, prepared from HSV-1 infected cells. This antigen does not differentiate antibodies to HSV-1 from antibodies against HSV-2. The antigen used for the detection of HSV-2 antibodies is a Helix pomatia lectin purified, type specific, membrane associated antigen prepared from HSV-2 infected cells and it migrates in polyacrylamide gels as a single band of 130 000 daltons. It is identical to the HSV-2 specific glycoprotein gG-2 (14). Sera from patients with verified HSV-2 infection cross react in approximately 75 per cent of the cases with the HSV-1 antigen. The antigens used for assay of antibodies against VZV and CMV were prepared as described earlier (15,16).

Immunoblotting.

Two ml of [^{35}S]-methionin-labeled HSV-1 infected HEp-2 cell extract was separated in 9.25% polyacrylamide gels according to Morse et al. (17). The multiplicity of infection was 40 plaque forming units per cell. The proteins were transferred to nitrocellulose papers (pore size 0.45 μm) by use of a transblot cell (Bio-Rad, Richmond, Ca., USA) (18). The transfer was cut in 0.5 cm wide strips. The strips were placed in an incubation tray (Bio-Rad, Richmond, Ca., USA). Each strip was washed three times in 0.05% Tween 20 in PBS and treated for 30 min with PBS containing 3% BSA, 4% FCS and 0.05% Tween 20. Each serum was dispersed in PBS supplemented with 3% BSA, 4% FCS, and 0.05% Tween 20, to a dilution of 1:100. Incubation time was 18 h followed by three washes in PBS with 0.05% Tween 20. Thereafter strips were incubated with horseradish peroxidase-coupled rabbit anti-human IgG (DAKO, Copenhagen, Catalogue No. P214) for 2 h using a 1:200 dilution of the conjugate in PBS with 3% BSA 4% FCS and 0.05% Tween 20. The antibody binding was visualized after wash and the addition of 4-chloro-1-naphtol (Bio-rad, Richmond, Ca., USA) (18). Each serum was tested on two different strips on two different occasions and all sera were analysed parallelly. Reactivity against different infected cell proteins (ICP) was identified and named in accordance with Morse et al. 1978 (17). The ICPs that could be detected with accuracy in this system was 4, gC, 11 (gA/gB), 22 (gE), and 29 (gD). Sera not reactive against ICP 4 at dilution 1:100 were also tested at dilution 1:20.

Statistical methods.

Data are presented as mean ± SE. The statistical significance of differences between group means were calculated with the Student's t-test and the significance of differences in proportions between groups was calculated with the Fischer's exact test and the chi square test. A p-value less than 0.05 was considered statistically significant (19).

RESULTS

Antibody Prevalence and IgG Titers against HSV, HSV-2, CMV and VZV.

No statistically significant differences in IgG antibody prevalence to any of the viruses tested were found between the cancer patients and the controls (Table 2). Of the 169 patients 91 % reacted against HSV in the ELISA.

The average log titers against HSV, HSV-2, CMV, and VZV are presented in Table 3. Antibody titers against HSV were signficantly higher in all groups of cancer patients compared to the control group. The highest titers were found in the groups of oral and laryngeal cancers. Antibody titers against HSV-2 and VZV did not differ significantly between the groups. In contrast to HSV, the antibody titers against CMV were significantly higher in the control group compared to the cancer patients.

Immunoblotting.

Antibody reactivity against ICP 4, gC, gB, gE, and gD is presented in Table 4. Fig. 1 shows the immunoblot of two sera from each group. Reactivity was found to several HSV-proteins but variability among individual sera was observed. One control serum and one serum from a laryngeal cancer patient that both were negative in the HSV ELISA were found to be positive in the immunoblotting to HSV proteins. In general, sera from cancer patients reacted to more HSV proteins than did the control sera. This difference was most pronounced in reactivity to the immediate early protein ICP 4. More than 80 per cent of sera from cancer patients reacted against ICP 4, compared to 53 per cent of control sera (Table 4). When sera, initially not reactive against ICP 4, were retested at a higher concentration (diluted 1:20 versus 1:100), no additional sera reacting with ICP 4 were found.

DISCUSSION

In the present study no difference in prevalence of antibodies against HSV between cancer patients and controls was found. Neither did the prevalence of antibodies against HSV-2, VZV nor CMV differ significantly. The prevalence of HSV antibodies in both groups was unexpectedly high (about 90 per cent). The prevalence of anti HSV IgG among blood donors in Gothenburg Sweden (predominantly young males) is approximately 65 per cent (T. Bergström, personal communication). Although no difference in prevalence was found, the titers of antibodies against HSV-1 were significantly higher in the cancer patients, particularly in the patients with oral and laryngeal cancers. There might be several explanations for this result. One is that the tumor elicits a general increase in the humoral immune response. However, the titers against VZV did not differ between cancer patients and controls. Furthermore, the titers against CMV were significantly lower in the cancer patients than in the controls. Another reason might be that cancer patients are more susceptible to reactivation of latent HSV infections, repeatedly triggering the immune response. We made an effort to inquire for the patients' history of herpetic lip lesions but it was impossible to obtain any reliable data of such. A third explanation is that the tumor development is associated with a different course of an earlier herpetic infection with a more intense or prolonged antigen challenge. Such a mechanism would not affect the IgG titers to other viruses of the herpes virus group. This does not exclude that tumors might contain a partly expressed or an incomplete but active HSV genome continously stimulating the IgG response to HSV (20-22).

In the present study immunoblotting revealed that most sera bound strongly to several HSV proteins, but inter-individual variability was observed. However, the reactivity against the different HSV proteins was more varied in the control patients than in patients with tumors. Especially reactivity against the immediate early protein ICP 4 was more frequently observed in cancer patients than in controls. Kassim and Daley (20) have reported that some tissue specimens from oral cancers stained positively when incubated with polyclonal anti-HSV serum and concluded that some oral cancers might develop due to an active part of the HSV genome in the host cell. Shillitoe et al.(23) found that sera from oral cancer patients more frequently reacted to two late HSV antigens recognized by IgM and IgA, respectively. Studies of reactivity against specific HSV-proteins in patients with preinvasive and invasive carcinoma of the uterine cervix have not revealed any specific pattern related to the disease (24). However, an antigen prepared from cells infected for only 4 h with HSV-2 (4, 25), is associated with the prognosis of cancer in the uterine cervix (26).

Tobacco interferes with the replicative cycle of HSV (27-29). Recently we have shown that immediate early HSV proteins ICP 4 and 27 are increased, and that cellular functions, as monitored by actin synthesis, are protected in HSV infected cells in the presence of tobacco extracts (P-A Larsson et al. submitted). One explanation of the high frequency of anti ICP 4 IgG reactivity in the cancer patients, who are predominantly smokers, might be that in these patients the exposure to tobacco has resulted in an increased exposure to immediate early HSV proteins. If so, the implications of such an increased exposure to a trans activating protein, as the HSV ICP 4, warrants further study.

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Table 1. Smoking habits in the different groups (%).

GROUP n	CONTROLS (79)	ORAL CANCER (28)	LARYNGEAL CANCER (16)	OTHER H/N CANCER (46)
Patients smoking > 20 cigarettes/day	68.4	46.4	25.0	32.6
Patients smoking 2-20 cigarettes/day	0.0	21.4	25.0	13.0
Patients using both smoked and/smokeless tobacco	5.1	0.0	6.2	0.0
Snuff dippers	1.2	0.0	0.0	6.5
Earlier tobacco users (more than one year with no tobacco consumption)	15.2	10.7	43.8	17.4
Non tobacco users	10.1	21.4	0.0	30.4

Table 2. Prevalens of IgG antibodies against HSV, HSV-2 CMV and VZV in the different groups of cancer patients and controls. Numbers of patients and percent.

	CONTROL		ORAL CANCER		LARYNGEAL CANCER		OTHER H/N CANCER		All CANCER	
	#	%	#	%	#	%	#	%	#	%
CMV	55	69.6	23	82.1	11	68.8	33	71.7	67	74.4
VZV	69	87.4	27	96.4	15	93.7	40	87.0	82	91.1
HSV	73	92.4	24	85.7	14	87.5	43	93.4	81	90.0
HSV-2	17	21.5	3	10.7	3	18.8	7	15.2	13	14.4

Table 3. Log titers for IgG-antibodies against HSV-1, HSV-2, CMV and VZV. Mean ± SE. * Denotes statistically significant difference compared to controls.

GROUP n	CONTROL (79)	ORAL CANCER (28)	LARYNGEAL CANCER (16)	OTHER H/N CANCER (46)	ALL CANCER (90)
HSV	3.84±0.062	4.30±0.093*	4.33±0.122*	4.09±0.093*	4.19±0.062*
HSV-2	2.78±0.112	3.30±0.099	2.90±0.000	2.80±0.197	2.99±0.107
CMV	2.85±0.066	2.52±0.078*	2.52±0.129*	2.60±0.068*	2.56±0.046*
VZV	2.21±0.058	2.31±0.057	2.24±0.044	2.25±0.042	2.11±0.129

Table 4. Specific reactivity in immunoblotting against different HSV-1 proteins. Numbers of patients and percent of sera reacting.

GROUP		CONTROL		ORAL CANCER		LARYNGEAL CANCER		OTHER H/N CANCER	
n		(79)		(28)		(16)		(46)	
mw		#	%	#	%	#	%	#	%
163	ICP 4	42	53	21	75	14	88	38	83
128	gC	64	81	24	86	15	94	41	89
114	gA/gB	67	85	24	86	15	94	43	93
70	gE	67	85	24	86	15	94	43	93
59	gD	64	81	24	86	15	94	37	80

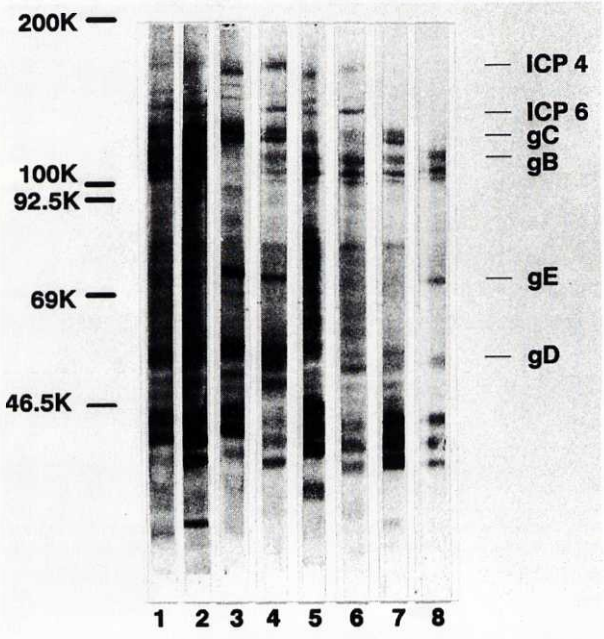

Fig. 1. Immunoblotting of human sera from patients with oral cancer (slot 1 and 2), laryngeal cancer (slot 3 and 4), lip cancer (slot 5and 6), and controls (slots 7 and 8). Position of the molecular weight marker is indicated to the left and the different HSV proteins to the right.

Printed in Sweden ✪ Goterna, Kungälv 1990.46893