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

Per-Anders Larsson

1990

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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)

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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.

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To Elisabeth and Malin

CONTENTS

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.

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