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Effects of Long-term Administration of Cancer-promoting Substances on Oral Subepithelial Mast Cells in the Rat

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Abstract. *The role of oral subepithelial mast cells in the defence against tumours is a matter of controversy. The effect of established and suggested carcinogens, such as the carcinogen 4-nitroquinoline-N-oxide (4-NQO) and Herpes simplex virus type 1 (HSV-1), in combination with oral snuff on lower lip subepithelial mast cells (MC) was studied in rats. The rats were exposed to prolonged use of oral snuff. The test substances were administered in a surgically created canal in the lower lip of the rats. There were 15 rats in each test group and 10 rats in the control group. The amount of countable subepithelial mast cells decreased significantly when the rat oral mucosa was exposed to the oral carcinogen 4-NQO but the effect of oral snuff and HSV-1 infection was weak. Our findings suggest that mast cells play a role in immunological cell defence against chemical carcinogens. Further studies are needed to clarify the mechanisms.*

The mast cell is found in all connective tissues, including the lamina propria of the oral mucosa, and upon stimulation it releases different biologically active substances such as neutral proteases, arachidonic acid metabolites, histamine, heparin and chemotactic factors for neutrophils and eosinophils. Thus, the mast cell has a primary role in the body's response to injury, both in the initiation of inflammation and in the subsequent process of repair (1). The mast cell population of oral mucosal tissues in rats contains both connective tissue mast cells and mucosal mast cells (2). Mast cells have been suggested to be involved in tumours of various characteristics and in various sites but the role of mast cells in tumour development and defence against tumours is still a matter of controversy (3-6).

Different carcinogenic substances have been investigated to induce cancer in experimental studies. The carcinogenic effect of 4-nitroquinoline-N-oxide (4-NQO) was first reported by Nakahara *et al.* in 1957 and successful induction of oral malignant squamous cell tumours was reported in 1965 (7, 8). An experimental model for oral cancer was described by Wallenius and Lekholm (9), who produced squamous cell carcinomas of the palatal mucosa in rats by repeated application of 4-NQO. 4-NQO has been used by Thomas *et al.* (10) to study oral tumour association with inflammatory infiltrate in rats. 4-NQO is not a carcinogen but a precarcinogen, *i.e.* it has to be metabolised to the ultimate carcinogen, which binds covalently to DNA, particularly to guanine and adenine (11, 12). Further, it is specific for base-pair substitutions (90%), principally G to A transitions. 4-NQO has also been suggested to activate proto-oncogenes by point mutation (13). Ha-ras mutations in oral rat mucosa have been detected after treatment with 4-NQO (14).

Snuff dipping, which affects the general as well as the oral health, results in characteristic intraoral mucosal lesions in both rats and humans (15, 16). Snuff may be of importance for HSV-1-induced transformation and the development of HSV-1-associated tumours (17). The incidence of experimental oral tumours and the histopathological effects on the oral mucosa in rats after initiation with 4-NQO and exposure to snuff and HSV-1 has been reported in a previous paper (18).

4-NQO is a well-characterized substance for tumour induction, while snuff and HSV-1 are suggested etiological agents in the development of oral squamous cell transformation. The aim of this study was to investigate the local effect of these substances on the amount of subepithelial mast cells in the oral mucosa of the rat.

Materials and Methods

Animals. Male, inbred Sprague Dawley and Lewis rats, purchased from Anticimex AB, Stockholm, Sweden, were used. The rats were 3 months old, with an average weight of 400 g, when the experiment started. Three to four rats were kept in macrolon cages (NO 3, Jacoby, Stockholm, Sweden) with hardwood bedding. The animals were fed a standard pelleted diet (Astra Ewos AB, Södertälje, Sweden) and tap water *ad libitum*. The temperature was kept constant between 21° and 23°C and the relative humidity was 40%. Light followed the diurnal rhythm and was never less than 8 hours.

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Surgical procedure. The rats underwent a surgical procedure to create a canal in the lower lip, which functioned as a reservoir for the test substances (16). In short, an excision of the mucous membrane was made extending from lateral to the lower incisors to 3 mm dorsal to the midline of the lip on both sides. The edges of the wound of the oral mucosa were sutured tightly. This procedure resulted in a test canal covered with mucous membrane. The operation was followed by a 10-day healing phase, after which the experimental treatment began. The study was approved by the ethics committee of Goteborg University, Gothenburg, Sweden.

Snuff. A commercially available Swedish brand was used in the study. Analyses of the average concentration of tobacco-specific N-nitrosamines (TSNA) in this brand have been published earlier (16). The snuff was applied in the test canal at 8 am and 4:30 pm 5 days a week by means of a plastic syringe. The test canal was completely filled, which on average meant the application of 200 mg of snuff. The average exposure time was 12 hours per day (16).

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

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 %.

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.

Group I, comprising 10 rats, served as a control group and was treated with propyleneglycol on a cotton swab once weekly for 5 weeks.

Group II, comprising 15 rats, received snuff 5 days a week in the test canal until killed.

Group III, comprising 15 rats, received 0.5 ml of HSV-1 suspension, 25,000,000 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 (19).

Group IV, comprising 15 rats, was treated with HSV-1 identically to Group III except that the treatment also included snuff administration identical to Group II, apart for 1 day each month when the virus inoculation was performed.

Group V, comprising 15 rats, received 4-NQO once weekly for 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 placed in the test canal for 24 hours and then removed.

Group VI, comprising 15 rats, was treated with 4-NQO identically to Group V for 5 weeks followed by snuff administration 5 days a week, identically to Groups II and IV.

Two rats in Group III died from encephalitis after 21 and 27 weeks, respectively, and were excluded from the study. Another rat in Group III, 2 rats in Group I, 2 rats in Group II, 3 rats in Group V and 3 rats in Group VI were found dead and suffered from pronounced autolysis so were therefore excluded from the experiment. The remaining rats constituted the effective number of test animals. The animals were killed after approximately 23 months by injection of phenobarbitone followed by exsanguination until cardiac arrest.

Histopathological methods. After gross external inspection, all the animals underwent autopsy. Specimens from the test canal were taken for light microscopic examination. Tisue 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. Another section of the paraffin-embedded tissue specimen was stained with 0.5% toluidine blue in acetate buffer, pH 4.0 (20). Staining with toluidine blue gave a light blue background, which permitted mapping of the metachromatic mast cells in relation to the other tissue components within the specimen. This staining was used for identification and counting of the mast cells.

Letraset(®) with 1 mm^2 squares was placed over one section for each specimen and the number of mast cells was counted in the subepithelial layer. The mast cells were counted in four separate squares in one section in close association to the epithelium. The mean of the 4 counts was calculated and comparison between the study groups was made with the Student's *t*-test.

Results

Mast cell number in the mucosa. The control group had a mean of 39.4 (SEM=5.8) MC/mm^2. The groups treated with snuff only and HSV-1 only had a mean of 39.0 (SEM=1.2) MC/mm^2 and 33.4 (SEM=1.7) MC/mm^2, respectively. The group treated with snuff/HSV-1 had a mean of 41.3 (SEM=2.5) MC/mm^2 (Figure 2). The 4-NQO-group (Figure 3) and the 4-NQO/snuff-group had a mean of 23.9 (SEM=1.2) MC/mm^2 and 30.6 (SEM=3.4) MC/mm^2, respectively (Table I). The only statistically significant differences found were between the 4-NQO-group and the control group ($p<0.01$) (Figure 1). The rats with head and neck tumours, irrespective of treatment, had a mean of 30.8 (SEM=3.0) MC/mm^2 in the test canal (Table II) and even though there was a tendency towards decreased amount of mast cells, this difference was not statistically significant compared to the control group.

Tumour incidence. Six oral squamous cell carcinomas were found in six rats. Three of these were growing in close proximity to the entrance of, but not in, the test canal. Squamous cell carcinomas, located in the crevicular epithelium close to the orifice of the lip canal, were found in the other three rats. Three rats had extraoral cancer in the head and neck region and one of these also had oral cancer. Thus, a total of eight rats had cancer in the head and neck region (Table II).

Preneoplastic lesions and reactive changes. Dysplasia of the squamous epithelium on the lip and in the crevicular epithelium was seen in 4 rats in three of the groups (Table II). Hyperplastic lesions were most prevalent in the 4-NQO/snuff-group but they were not confined to the test canal.

Discussion

In a study by Larsson *et al.,* an animal model was used to investigate and compare the role of oral snuff, HSV-1 and 4-NQO alone or jointly in tumour development (18). We have explored this model to investigate the effects of 4-NQO, HSV-1 infection and oral snuff on this putative defence system against tumours. In the present study, we found that rats treated with 4-NQO had a significantly lower amount of mast cells (23.9 MC/mm^2) in the test canal compared to the control group (39.4 MC/mm^2). The group treated with 4-

Table I. *Number of animals (%) displaying dysplasia and cancer.*

	Control n=8	4-NQO n=12	Snuff n=13	HSV n=12	Snuff/ HSV n=15	Snuff 4-NQO n=12
Dysplasia	0	0	2(15)	1(8)	1(7)	0
Cancer	0	(2(17)	1(8)	2(15)	1(7)	2(17)

Table II. *Group, number of mast cells per mm^2 and diagnosis of the rats with dysplasia or cancer in the head and neck region.*

Group	Number of MC/mm^2	Diagnosis
HSV-1	30.5	Cancer
HSV-1	38.5	Cancer
HSV-1	40.8	Dysplasia
HSV-1/snuff	15.8	Dysplasia
HSV-1/snuff	44.8	Cancer
Snuff	33.8	Cancer
Snuff	44.8	Dysplasia
Snuff	41.2	Dysplasia
4-NQO	31.2	Cancer
4-NQO	21.2	Cancer
4-NQO/snuff	18.5	Cancer
4-NQO/snuff	28.2	Cancer

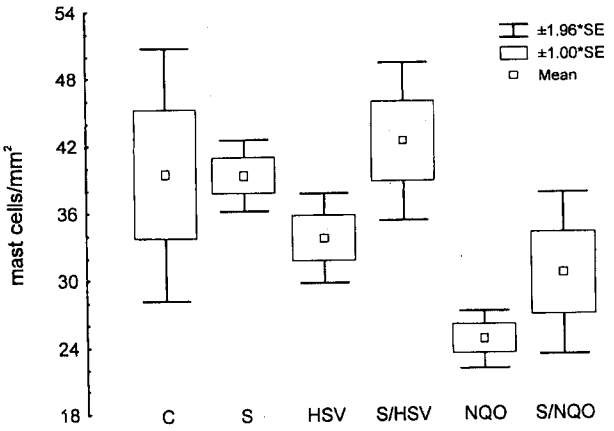

Figure 1. *Amount of mast cells/mm^2 in the different groups.*

NQO/snuff also had a tendency to fewer mast cells in the subepithelial layer (30.6 MC/mm^2), although the reduction was not statistically significant. Our observations demonstrated that there is a negative correlation between 4-NQO and the number of countable mast cells.

Farram and Nelson (21) showed that mouse mast cells *in vitro* have the ability to kill tumour cells, probably by means of a mediator. Infiltration of mast cells as a response to an immunological stimulus can conceivably lead to tumour cell destruction by the potent mediators in the mast cells. On the other hand, high mast cell activity governs cell proliferation in the surrounding tissue, which may be a cancer-promoting mechanism, according to Norrby (22) and earlier studies have also shown that the presence of mast cells may facilitate the process of tumour invasion by promoting angiogenesis (23). Matthews *et al.* (24) also found that, although immune reactions are stimulated by 4-NQO treatment of rat oral mucosa, the effector cells necessary for controlling tumour development and growth are absent.

The number of mast cells along the tumour-host junction is reported to be significantly greater in the beginning of tumour formation (4). Flynn *et al.* (25), who studied mast cell density in hamster buccal pouches after treatment with the carcinogen DMBA, found a positive correlation between mast cell density and developing carcinomas. No data is available regarding long-term exposure to carcinogenic agents. That study involved 4-months' exposure to DMBA, compared to an average of 23 months of 4-NQO exposure in the present study suggesting that the length of exposure to chemical carcinogens may influence the mast cell population. Mast cells have also been suggested to decrease in the oral mucosa in adult rats (26). In our study however, no age-related decrease in mast cell population was seen between the different groups.

Tanooka *et al.* (27) showed that mast cell-deficient mice had an increased tumour incidence after treatment with a carcinogenic agent and suggested that mast cells are involved in tumour suppression. Lachter *et al.* (28) showed that the mast cell numbers were significantly decreased in premalignant disorders and malignant neoplasias in colorectal lesions compared to normal colorectal tissue. Tumour induction after 4-NQO treatment may be enhanced by this mechanism. These studies support our present results although other studies have suggested that mast cells have little or no correlation with tumour development or tumour prognosis (29).

It must, however, be noted that the decrease of stained mast cells in the present study could represent a true decrease in mast cell numbers in the tissue, but it could also reflect an increased activity with degranulation of the mast cells. The dye used stains the mast cell granule and advanced

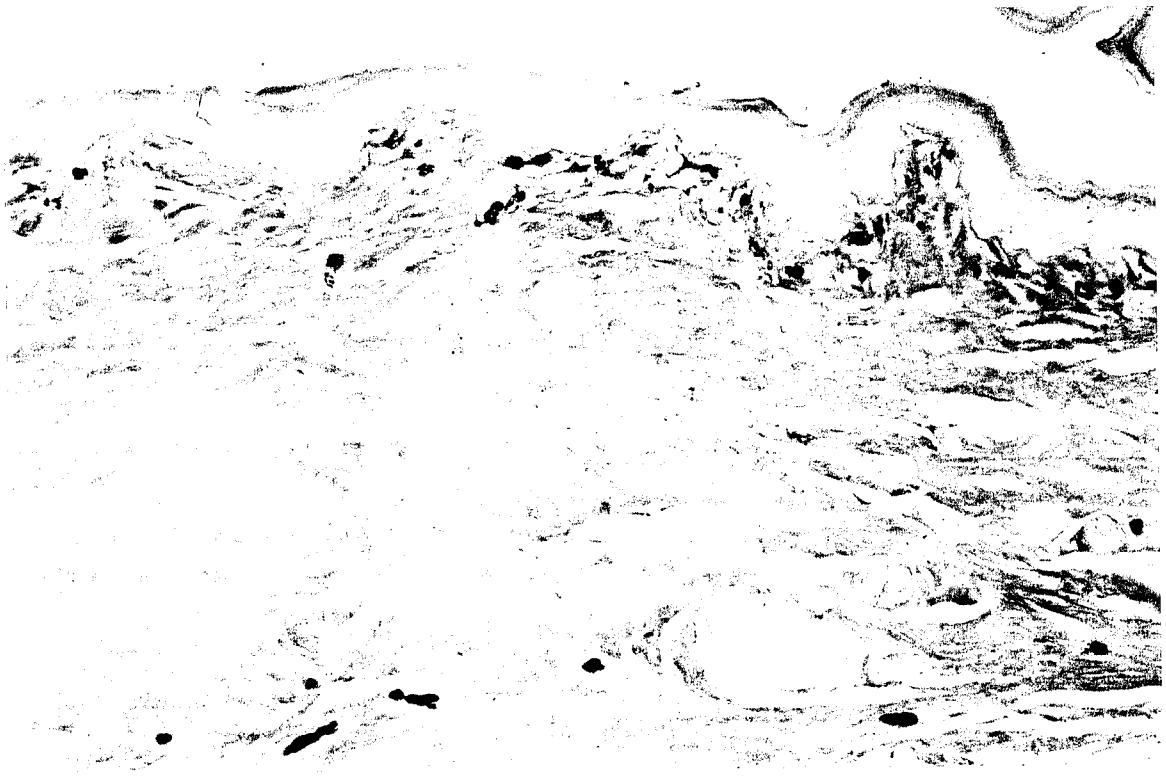

Figure 2. *Dark-blue staining cells of variable shape in the test canal in a rat exposed to snuff and HSV-1 for 22 months. (Original magnification, x400).*

Figure 3. *Light-microscopic appearance of the test canal in a rat exposed to 4-NQO for 20 months. Scattered mast cells are visible with toluidine blue staining in the subepithelial layer. (Original magnification, x400).*

degranulation of (activated) mast cells may not be identified. Techniques with electron microscopy were not used in this study. Activated mast cells have been shown by Dabbous *et al.* (30) to release substances like histamine and heparin that promote tumour growth while in a study by Hartveit *et al.* (31), mast cell degranulation was a characteristic of areas of infiltrative growth in human breast carcinomas.

In conclusion, this study has shown that the carcinogenic substance 4-NQO significantly influences the mast cell population. Our findings suggest that mast cells may play an important role in immunological cell defence against chemical carcinogens. However, further studies are necessary to clarify the mechanisms and whether the decrease in mast cell population is a true decrease or simply an increased activation of the mast cells. Changes in mast cell population were not detected with the other substances applied, indicating less efficient carcinogens or different cancer-promoting mechanisms.

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