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Immunotoxic effects of smokeless tobacco on the accessory cell function of rat oral epithelium

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Smokeless tobacco (ST) is known to adversely effect the oral mucosa, but knowledge about the influence on immune defence is limited. Few studies have investigated the effect of ST on the local immune response. In the present study, we have assessed the effect of a crude Swedish moist snuff (SS) extract, alkaloids, and nitrosamines on T-cell mitogenic response to Con A using epithelial cells, including Langerhans cells, of the rat oral mucosa as accessory cells. SS extract at a concentration of 4% reduced the T-cell proliferation by 50% (IC_{50}=4%). Pretreatment of either oral epithelial cells or T-cells with SS extract also gave a significant inhibition of T-cell proliferation. This effect was not obtained following preincubation with SS components as alkaloids and different tobacco-specific nitrosamines (TSNA). None of the tested compounds were found to possess any mitogenic properties. This in vitro study showed that SS extract can evoke an immunosuppressive effect on mitogen-driven T-cell proliferation using cells from oral epithelium as accessory cells. This effect was more pronounced when SS extract was employed compared to when the single SS components were used alone.

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Various toxicological reactions in the oral mucosa have been related to the use of smokeless tobacco (ST) (1). Clinical studies designed to investigate mucosal changes have revealed that the use of moist snuff causes parakeratinization, acanthosis, stromal inflammation, and degenerative changes in the minor salivary glands (2–4). Equivalent toxicological reactions to those seen in man have also been observed following experimental studies in laboratory animals (5–7). Studies in vitro have shown that ST extracts has an immunostimulatory potential (8), but have also been found to depress lymphokine-activated killer-cell activity (9). Furthermore, NK-cells in peripheral blood from rats exposed to oral snuff showed decreased activity in vitro (10).

In morphological studies, ST exposure has been demonstrated to affect the immune system by reducing the number of Langerhans cells (LC) in the oral mucosa (11). LC represent a peripheral outpost and provide the oral mucosa with an immunosurveillant competence (12). LC have the ability to internalize and process antigens, and after migration to the regional lymph nodes, are able to execute their accessory cell function and present antigens to naive T-cells (13). Upon stimulation, T-cells clonally expand, leave the lymph nodes and find the site of antigen entrance where these cells orchestrate the local immune response (14).

As it is reasonable to assume that the immune system of the oral mucosa at the site of ST exposure could be affected, we have used a previously developed method (15) to evaluate how commercial Swedish moist snuff (SS) and some of its derivatives affect the functional capacity of accessory cells from rat oral epithelium.

Materials and methods

Animals and tissue preparation

In experiments with epithelial cells and T-cells, 15–30 inbred 8–10-week-old Lewis rats were used. Spleen cells were also obtained from these animals for experiments to establish suitable concentration ranges for the different materials used. The rats were sacrificed by exposure to saturated CO_2 and cervical dislocation. Cervical or mesenteric lymph nodes were dissected and pooled; spleens were removed by blunt dissection after incision through the ventral abdomen. Oral mucosa was obtained by dissecting the buccal mucosa from the underlying musculature. All tissue specimens were pooled in cold Dulbecco's modification of Eagle's medium containing gentamycin (20 mg/l; DMEM+).

Preparation of cells

Spleen cells – Spleens, kept in a Petri dish with DMEM+ containing 10% heat-inactivated foetal calf serum (DMEM++), were gently squeezed with forceps to release the spleen cells. The released cells were collected and washed twice at ×400*g*. To remove dead cells and erythrocytes, the cell suspension was subjected to a density gradient centrifugation (Ficoll-Paque; Pharmacia Fine Chemicals, Uppsala, Sweden) according to the manufacturer's instructions. The cell suspension was washed twice in DMEM++ and cell viability was estimated by trypan blue exclusion. Cell viability was never found to be below 85%.

T-cells – Dissected mesenteric or cervical lymph nodes were gently disrupted with forceps. Cells were suspended and washed in DMEM++. As for spleen cells, red blood cells and dead cells were removed following density gradient centrifugation. MHC class II molecule-expressing cells, i.e., macrophages, B cells and dendritic cells normally present in the lymph node, were removed by the following procedure: (i) incubation overnight at 4°C with a mouse monoclonal IgG anti-rat MHC class II molecule (1:100, Ox6; Sera-lab, Sussex, UK); (ii) repeated washes in cold DMEM++ to remove free antibodies; (iii) incubation for 1 h at 37°C with immunomagnetic beads coated with goat anti-mouse-IgG (cell/bead ratio of 1:20; Dynabeads M-280; Dynal, Oslo, Norway), and (iv) separation of MHC class II molecule-expressing cells (rosetted with beads) from non-rosetted T-cells with a magnet. This was followed by several washes of the purified T-cells in cold DMEM++. In later experiments, the purification protocol of T-cells was modified by the introduction of a nylon wool column (16) prior to the treatment with antibodies and beads described above. Cell viability was estimated by trypan blue exclusion and viability consistently exceeded 95%. Immunohistochemistry of purified T-cells revealed virtually no MHC class II molecule-expressing cells in the cell suspension.

Epithelial cells – Dissected mucosal membranes were kept in 0.2% chlorhexidine solution (Hibitane Dental; ICI-Pharma, Stockholm, Sweden) for 5 s and then transferred to DMEM+. Membranes were floated on 20 mM EDTA (Na_4-EDTA; Kebo, Stockholm, Sweden) and incubated at 37°C for 2 h in 5% CO_2 atmosphere. Epithelium was separated from subepithelial tissue by fine forceps. Epithelial sheets were spread on a Petri dish with the subepithelial side upwards and covered with 0.5% trypsin (Life Technologies, Paisley, UK) and further incubated at 37°C for 30 min in 5% CO_2 atmosphere. Immediately after this incubation procedure, epithelial sheets were covered with DMEM++ supplemented with 20% FCS to inactivate the trypsin. To release epithelial cells, sheets were first transferred to a vial containing fresh DMEM++ and then subjected to gentle pipetting. After sedimentation for 5 min, a supernatant containing released epithelial cells was harvested. The cell suspension was washed twice in DMEM++, and cell viability was estimated by trypan blue exclusion.

SS-extract, alkaloids and nitrosamines

SS-extract – Commercially available Swedish moist snuff (brand Röda Lacket; Svenska Tobaksbolaget, Göteborg, Sweden) was used and SS extract was prepared as described earlier (17). Briefly, 10 g of SS dissolved in 50 ml DMEM++ was incubated in 37°C for 1 h. The solution was centrifuged twice at ×400 *g* for 10 min to remove undissolved tobacco. The obtained supernatant was kept frozen at −76°C until further use. Before experimentation, pH was adjusted to 7.2 with 0.1 M HCl and subjected to sterile filtration through 0.2 μm disposable sterile filter (Acrodisc; Gelman Sciences, Ann Arbor, MI, USA).

Alkaloids and nitrosamines – Anabasine (ANA; 100 μg/g), N'-nitrosoanabasine (NAB; 0,20 mg/kg), nicotine (NIC; 10 mg/g), N'-nitrosonornicotine (NNN; 2.5 mg/kg), 4-(N-nitrosomethylamino-1-(3-pyridyl)-1-butanone (NNK; 0.80 mg/kg) and N-nitrosdimethylamine (NDMA; 0.001 mg/kg) are all present in Swedish moist snuff at given concentrations (18–20). The alkaloids and TSNA are commercially available (Chemsyn Science Laboratories, Lenexa, KA, USA and Sigma Chemical Co., St. Louis, MO, USA) and have been used in previous studies to assess their influence on

cell growth and differentiation (21–23). To obtain concentrations of alkaloids and TSNA which correspond to those found in SS, NAB, NNN, NNK, ANA, and NDMA were diluted in DMEM++. The pH of the prepared solutions was the same as for DMEM++ (pH 7.6). Dilution of NIC to a concentration which corresponded to 10 mg/g gave a pH>9. Therefore NIC was diluted to a concentration 0.001 mg/g (1:10000) to obtain a physiological pH suitable for cell culturing. This solution, although not corresponding to the concentration of NIC in SS, was used in some tests. All stock solutions were kept frozen in vials at −76°C until further use. Before experimentation, the solutions were sterilized by filtration through 0.2 μm disposable sterile filter (Acrodisc). Whenever possible, test solutions were kept in the dark to avoid photochemically induced reactions.

Functional assays

Spleen cells – In toxicity experiments, spleen cells were transferred to 96-well U-bottom culture plates (Costar, Cambridge, MA, USA) at 2.5×10^5 cells/well in 100 μl of DMEM++. SS-extract (0.2%, 0.8%, 3%, 12.5% and 50% extract concentration in culture wells), alkaloids or TSNA (concentrations in culture wells corresponding to concentrations in SS as described under *Alkaloids and Nitrosamines*) in a volume of 100 μl were added to each well. Triplicates were run and to each well 5 μg/ml Con A (Pharmacia LKB Biotechnology, Uppsala, Sweden) was added. In control wells, test solutions were replaced by 100 μl DMEM++. Incubations were performed at 37°C for 72 h in 5% CO_2 atmosphere. Following 48 h of incubation, 5 μCi/ml of [^{3}H]-thymidine (methyl-[^{3}H]-thymidine; 24 Ci/nmol, Amersham, Bucks., England) was added to each well. After an additional 24 h, the cells were harvested onto glass-fiber filters in a Skatron harvester (Flow Laboratories, Oslo, Norway) and radioactivity was counted in a liquid scintillator. All experiments were repeated at least three times.

In pretreatment experiments, the spleen cells were pretreated for 4 h at 37°C in 5% CO_2 atmosphere with SS-extract, alkaloids or TSNA dissolved in DMEM++ at concentrations given above. The cells were washed twice and cell viability was assessed by trypan blue exclusion. Viable cells (2.5×10^5/well) were incubated as previously described.

Epithelial cells and T-cells – In toxicity experiments, epithelial cells (EC; 2×10^4 cells/well) and T-cells (2.5×10^5 cells/well) were transferred in a volume of 100 μl DMEM++ to 96-well U-bottom culture plates (Costar). SS-extract, alkaloids or TSNA (concentrations in culture wells corresponding to concentrations in SS as described under *Alkaloids and Nitrosamines*) in a volume of 100 μl were added to each well. Control wells received only additional 100 μl DMEM++. To triplicate wells, 5 μg/ml Con A/well (Pharmacia LKB Biotechnology) was added. Incubations and cell harvesting were performed according to the protocol described for spleen cells.

In pretreatment experiments, the epithelial cells and the T-cells were pretreated for 4 h at 37°C in 5% CO_2 atmosphere with the various concentrations of SS-extract, alkaloids or TSNA. After repeated washes, cell viability was assessed by trypan blue exclusion and the cells were then transferred to 96-well U-bottom culture plates (Costar) and incubated in a cross over fashion; pretreated EC (2×10^4 cells/well) and untreated T-cells (2.5×10^5 cells/well); untreated EC (2×10^4 cells/well) and pretreated T-cells (2.5×10^5 cells/well); untreated EC (2×10^4 cells/well) and untreated T-cells (2.5×10^5 cells/well). Con A (5 μg/ml) was added and subsequent incubations and harvesting of cells were performed as described above.

Statistical methods – We express our data as mean values and standard deviation of three independent experiments. Due to the fact that cpm values varied between different experiments an internal standard was included in all experiments to accommodate for these variations. Hence, all data are expressed in percent of the proliferation rate in the absence of SS components. The cpm value of the internal standard never dropped below 20,000 cpm, and the proliferation of purified T-cells in the presence of Con A never exceeded 10% of this value. Statistical significance was calculated by analysis of variance (ANOVA) and using Fisher's Least Significant Difference Test for pairwise comparisons between groups as a posthoc test. A p-value of <0.05 was regarded as statistically significant. Computations were made using the statistical software Systat 5.2 (SYSTAT, Evanston, IL, USA).

Results

A suitable concentration range to study the cytotoxicity of the SS-extract was established by incubation of spleen cells with various concentrations of the extract in the presence of con A. A significant inhibition of spleen cell proliferation ($p<0.05$) was noted at a concentration of 0.8% (Fig. 1). Already at a concentration of 2%, the proliferation was reduced by 50% ($IC_{50}=2\%$). To investigate whether the spleen cells were able to recover from the

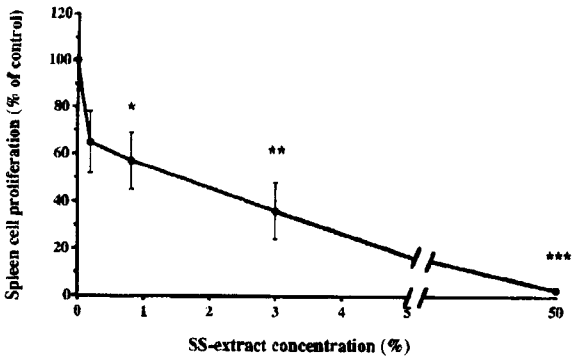

Fig. 1. Proliferative response of spleen cells (2.5×10^5 cells/well) when incubated with various concentrations of SS-extract and con A (5 μg/ml) in the culture wells. There is a concentration dependent inhibition of spleen cell proliferation. Already at a concentration of 0.8% extract there is a significant inhibition ($p<0.05$) of spleen cell proliferation. An extract concentration of 2% inhibits the proliferative response with 50% ($IC_{50}=2\%$). Data are expressed in % of cell proliferation of untreated spleen cells stimulated with con A (5 μg/ml), and given as mean values ±SEM of three separate experiments. $*p<0.05$; $**p<0.01$; $***p<0.001$.

cytotoxicity of the extract, 3 different concentrations of SS-extract were selected for preincubation experiments. Spleen cells were found to recover from SS-extract exposure in concentrations less than 6% (Fig. 2).

When epithelial cells, including Langerhans cells (LC), and T-cells were incubated with the various concentrations of the SS-extract, a similar response was obtained as noted for spleen cells. A significant inhibition of cell proliferation occurred at 12.5% ($p<0.05$; Fig. 3), and at a concentration of 4%, the T-cell proliferation was reduced by 50% ($IC_{50}=$

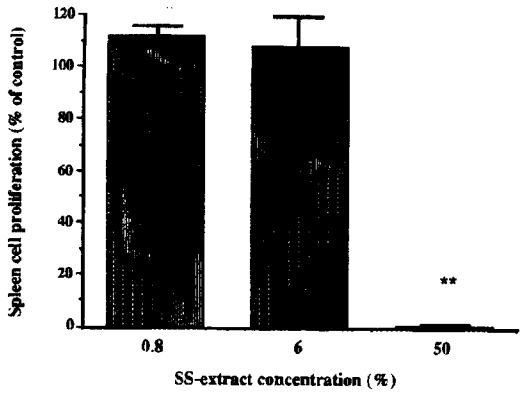

Fig. 2. Proliferative response of spleen cells (2.5×10^5 cells/well) after pretreatment with three various concentrations of SS-extract. After pretreatment, the spleen cells were stimulated with con A (5 μg/ml) in the culture wells. When pretreated with an SS-extract concentration of 50% the proliferative response was significantly inhibited ($p<0.01$). Data are expressed in % of cell proliferation of untreated spleen cells stimulated with con A (5 μg/ml), and given as mean values ±SEM of three separate experiments. $**p<0.01$.

4%). The ability of epithelial cells and T-cells to functionally recover from SS-extract exposure was tested by pretreatment with 3 different concentrations of SS-extract. The pretreated epithelial cells were then incubated with untreated T-cells and the pretreated T-cells with untreated epithelial cells. Both epithelial cells and T-cells recovered from the cytotoxic effects at a concentration less than 6% (Fig. 4). This concentration gave approximately a 60% reduction when present during the entire incubation (Fig. 3). Pretreatment of epithelial cells with an extract concentration of 50%, followed by culturing with untreated T-cells and con A, resulted in a significant permanent reduction of T-cell proliferation compared to when non-exposed epithelial cells were used as accessory cell ($p<0.001$; Fig.4). A similar reduction of T-cell proliferation was observed when T-cells were pretreated with an extract concentration of 50% and subsequently cultured with untreated epithelial cells and con A ($p<0.01$; Fig. 4).

To assess if alkaloids and tobacco-specific nitrosamines (TSNA) had the same effect as the SS-extract, ANA, NAB, NNN, NNK and NDMA were tested in concentrations calculated to be present in SS. In initial experiments, spleen cells were incubated with test substances in the presence of con A. None of these SS derivatives caused significant stimulation or inhibition of spleen cell proliferation compared to nonexposed cell populations (Fig. 5). The same results were obtained in experiments where spleen cells were preincubated with the highest available concentration of the test compounds (Fig. 5).

When the various alkaloids and TSNA were present during the entire incubation of epithelial cells and T-cells, no significant difference in cell proliferation was observed for any of the substances tested (data not shown). A negative outcome was also revealed after pretreatment of the two cell populations (Fig. 6). Although NNN showed a tendency to be stimulatory and NAB to be inhibitory, there were no significant differences between controls and pretreated cells (Fig. 6). Incubation and pretreatment of the various cell populations with the alkaloid NIC was also performed. The concentration tested was 1 : 10 000 of what is normally found in SS, as this was the only concentration where a pH suitable for cell culturing was achieved. At this concentration, no effect on cell proliferation was detected.

Incubation or pretreatment of spleen cells, epithelial cells and T-cells with the different test compunds in the absence of con A did not show any effect. Thus, no mitogenic capacity of the SS-extract, alkaloids and TSNA was revealed.

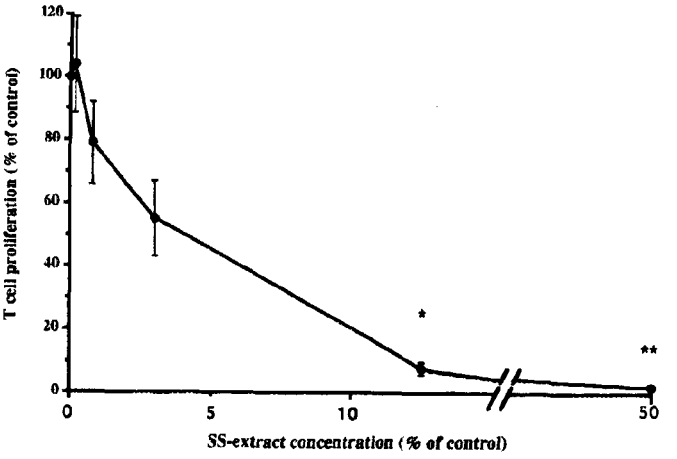

Fig. 3. Incubation of oral epithelial cells including LC (2×10^4 cells/well), and T-cells (2.5×10^5 cells/well) with various concentrations of SS-extract and con A (5 μg/ml). There is a concentration dependent inhibition of T-cell proliferation. A concentration of 12.5% ST-extract in the culture wells results in a significant inhibition ($p<0.05$) of T-cell proliferation. The proliferative response is reduced to 50% at a concentration of 4% ($IC_{50}=4\%$). Data are expressed in % of cell proliferation of oral epithelial cells including LC and T-cells stimulated with con A (5 μg/ml) but without SS-extract, and given as mean values ±SEM of three separate experiments. $*p<0.05$, $**p<0.01$.

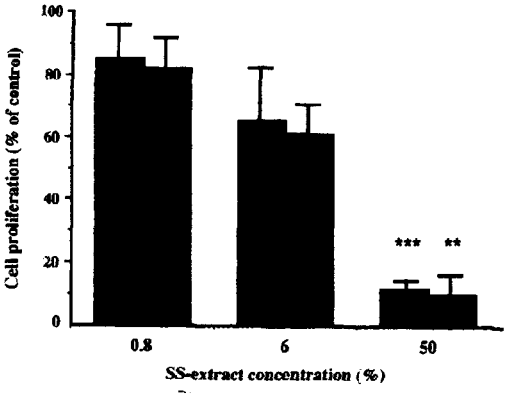

Fig. 4. Proliferative response of T-cells when pretreated epithelial cells, including LC (2×10^4 cells/well) were cultured with untreated T-cells (2.5×10^5 cells/well) (■) and pretreated T-cells cultured with untreated epithelial cells (▨) in the presence of three various concentrations of SS-extract and con A (5 μg/ml). Both epithelial cells and T-cells withstand an 6% ST-extract exposure. At a concentration level of 50% there is a significant reduction (epithelial cells: $p<0.001$; T-cells: $p<0.01$) of mitogen stimulated T-cell proliferation. Data are expressed in % of T-cell proliferation when untreated epithelial cells, including LC, were cultured with untreated T-cells stimulated with con A (5 μg/ml), and given as mean values ±SEM of four separate experiments. $**p<0.01$; $***p<0.001$.

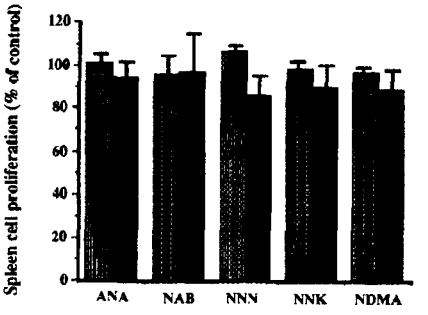

Fig. 5. Proliferative response of spleen cells (2.5×10^5 cells/well) when pretreated (▨) or incubated (■) with ANA, NAB, NNN, NNK, NDMA and con A (5 μg/ml). No significant effect ($p>0.05$) of alkaloid or TSNA exposure could be noted. Data are expressed in % of spleen cell proliferation in the absence of alkaloids or TSNA and given as mean values ±SEM of 4 separate experiments.

Discussion

The effect of smokeless tobacco (ST) has been investigated in several morphological and functional studies over the last three decades. Studies designed to assess the effect of ST on immunocompetent cells of the oral mucosa are more sparse. It has been reported that the number of Langerhans cells (LC) is reduced at the site of tobacco exposure but not in clinically normal mucosa adjacent to the tobacco-induced lesions (11). In the present study, the observation was made that water soluble extract from Swedish moist snuff (SS) significantly inhibited con A-stimulated T-cell proliferation induced by accessory cells from rat oral epithelium. When T-cells and oral epithelial cells were pretreated with high concentrations of the extract, there was a significant irreversible inhibition of T-cell proliferation. However, SS derivatives as alkaloids and tobacco-specific nitrosamines (TSNA) in concentrations as in SS had no significant effect. Furthermore, SS extract, alkaloids or TSNA did not show any mitogenic properties.

When present during the entire incubation, even low concentrations of SS extract inhibited T-cell

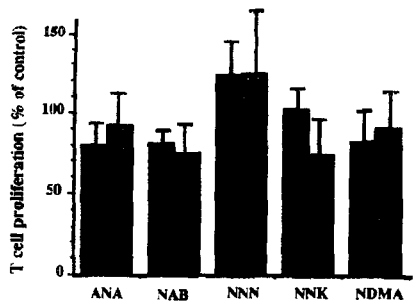

Fig. 6. Proliferative response of pretreated epithelial cells, including LC (2×10^4 cells/well) cultured with untreated T-cells (2.5×10^5 cells/well) (■) and pretreated T-cells (2.5×10^5 cells/well) cultured with untreated epithelial cells (2×10^4 cells/well) (▨) in the presence of ANA, NAB, NNN, NNK, NDMA and con A (5 μg/ml). There is no significant effect ($p > 0.05$) of pretreatment of epithelial cells or pretreatment of T-cells for any of the tested alkaloids or TSNA. Data are expressed in % of T-cell proliferation of untreated epithelial cells, including LC cultured with untreated T-cells stimulated with con A (5 μg/ml) and given as mean values ±SEM of 3 separate experiments.

proliferation. This observation concur with previous findings where phytohaemagglutinin-(PHA) induced mitogenesis decreased following exposure of ST to human peripheral lymphocytes (9). In the absence of mitogen, murine T-cells have been demonstrated to increase in proliferation at lower concentrations (0.05–0.5%) of ST extract than used in the present study (8). Thus, the effect of smokeless tobacco may vary from an irreversible cytotoxicity to a stimulatory effect observed at low concentrations. Most likely, these responses will also have a correlate *in vivo*. A concentration gradient may develop which causes irreversible cytotoxicity close to the applied SS, displayed as an atrophic or erosive lesion of the oral mucosa, while lower concentrations at the periphery may induce a proliferative response expressed as a hyperkeratinization and hyperplasia.

The association between the development of squamous cell carcinoma and habitual use of ST has been clinically (25) as well as experimentally (7) documented. In addition, in animal models it has been established that NNN and NNK (26, 27) have the potential to promote malignant transformation. It is possible that these ST constituents may operate through a cytotoxic effect on LC, as LC have been suggested to be involved in the protection of the oral mucosa from transforming into a squamous cell carcinoma (28) However, in the present study no cytotoxic effect on LC of the separate ST constituents were revealed. This observation does not exclude an effect on LC *in vivo*, caused by a synergistic action of TSNA and other components present in the SS extract. This hypothesis is substantiated by the observation that the SS extract was a more potent inhibitor of T-cell proliferation than the separate SS constituents. Thus, a chronic exposure *in vivo* to SS extract may interfere with the regulatory functions of Langerhans cells directed to control malignant transformation (28). As a sequel to this breakage of immuno-regulatory control, a microbiological invasion, by for example HSV-1, may be responsible for the observed increase of experimentally induced malignant tumors (5, 29).

Cytotoxic effects were disclosed when T-cells were preincubated with the extract, which resulted in a decreased proliferation of T-cells when these cells were further incubated with untreated oral epithelial cells. This implicates a cytotoxic effects on T-cells by the SS extract which could be of importance for capacity of the oral mucosa to instigate an immune response. Under healthy conditions, T memory cells patrol the subepithelial connective tissue and are responsible for orchestrating the secondary immune response when the oral mucosa is subjected to an antigenic challenge. The *in vivo* implication of this observation is that the exposure of SS to the oral mucosa may induce a local immunosuppression, not only through an effect on Langerhans cells but also on T-cells.

Most likely, long-term tobacco exposure will cause injury to different defence systems of the oral mucosa including the local immune system. An impaired immunosurveillant capacity by Langerhans cells following SS exposure, may open direct or indirect avenues for SS derivatives to cause various adverse effects. However, it was not possible in the present study to identify any specific SS component which have a more potent cytotoxic effect than others.

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