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Effect of Nicotine on Secretory Component Synthesis by Secretory Epithelial Cells

RICHARD L. GREGORY[1,2]* AND LINDA E. GFELL[1]

Department of Oral Biology[1] and Department of Pathology and Laboratory Medicine,[2] Indiana University, Indianapolis, Indiana 46202-5186

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Previously, we reported that secretory component (SC), lactoferrin (LF), and lysozyme (LY) levels were significantly lower in saliva from smokeless tobacco (ST) users than in saliva from control non-tobacco users. However, the levels of salivary immunoglobulin A were significantly higher, albeit with an altered attachment of SC, in ST users than in control subjects. SC, LF, and LY are synthesized by secretory epithelial cells at mucosal sites adjacent to lymphocyte regions. In the present report, HT-29 human epithelial cells, cultured with various concentrations of an ST aqueous extract or pure nicotine (0 to 1 mg/ml) or cotinine (0 to 5 mg/ml), exhibited significantly lower levels of cell-associated cell lysate (CL) and secreted culture supernatant (CS) SC, LF, and LY than cells cultured without ST components. Nicotine significantly decreased ($P \leq 0.05$) the synthesis of SC by 20 to 100%, LF by 20 to 60%, and LY by 5 to 75% of CL and CS control values. Studies also indicated significant decreases ($P \leq 0.05$) in SC, LF, and LY levels in both CL and CS of cells cultured with ST aqueous extract or cotinine. Total cell numbers and metabolic activity significantly decreased primarily when cells were incubated with higher concentrations of ST extract, nicotine, or cotinine. The addition of human recombinant interleukin-4 or gamma interferon diminished the effects ST had on HT-29 cell synthesis of SC, LF, and LY. Our data indicate that nicotine, cotinine, and ST have an adverse effect on synthesis and secretion of SC, LF, and LY. These effects were below ST concentrations found to be cytotoxic for secretory epithelial cells. Furthermore, addition of interleukin-4 or gamma interferon reduced the suppressive effect of ST on synthesis or secretion of SC, LF, or LY.

The principal immunoglobulin (Ig) in external secretions and in the entire body is IgA, and its role in protection against certain mucosal pathogens has been established (see reference 20 for review). Secretory IgA antibodies inhibit microbial adherence, colonization, and penetration of mucosal surfaces; inhibit metabolic pathways; neutralize enzymes, viruses, and toxins; mediate expulsion of plasmids and agglutination of microbes; and inhibit the growth of certain organisms (3, 12, 21, 22, 29 [see references 9 and 17 for review]). It is generally accepted that foreign antigens in the small intestine are sampled by microfolding cells in Peyer's patches and are subsequently passed to professional antigen-presenting cells which present antigenic determinants to IgA-precursor B and regulatory Th2 lymphocytes. These antigen-presenting, B, and Th2 cells then migrate through the lymphatics and blood circulation and home to the various effector mucosal immune tissues; salivary, lacrimal, and mammary glands; and the lamina propria regions of the gastrointestinal, upper respiratory, and genitourinary tracts. There the B lymphocytes differentiate into IgA-producing plasma cells and secrete specific IgA antibodies (8, 14, 30). Plasma cells at mucosal sites are located in proximity to secretory epithelial cells (SEC), which are responsible for producing several secretory products, including secretory component (SC), lactoferrin (LF), and lysozyme (LY [15, 16]). IgA leaving mucosal plasma cells is present as an IgA-J chain–IgA polymer and interacts with a polymeric Ig receptor (molecular mass of 95,000 Da) on the basolateral surfaces of SEC, where noncovalent binding occurs (see reference 18 for review). This receptor cross-reacts immunologically with SC and is believed to be SC with a 15,000-Da cytoplasmic extension anchor. SC serves as a polymeric Ig receptor for transcellular transport of IgA (or IgM) across the SEC. The complex is internalized and is stabilized by disulfide bridges. Epithelial cell proteolytic enzymes then cleave SC in the vesicle membrane-bound IgA-J chain–SC-IgA complex, and polymeric IgA is secreted at the apical SEC surface into saliva, tears, colostrum, milk, and mucous fluids (8, 14, 30 [for reviews see references 1, 4, and 19]). This results in an 80,000-Da SC bound to the IgA polymer and leaves a transmembrane form of SC of 15,000 Da which was originally responsible for anchoring the larger 95,000-Da SC form in the cytoplasm of the SEC. In addition, some SC is released as free SC and is found in detectable concentrations in secretions and serum. LF and LY play important roles in innate mucosal defense.

The incidence of smokeless tobacco (ST) use is increasing (5), and the potential effects on mucosal defense proteins are not well understood. Tobacco is composed of many pharmacological agents, including nicotine and its major metabolite, cotinine, that may affect the mucosa. Nicotine initially stimulates exocrine gland secretion, followed by an inhibition of secretion (26). Absorption of nicotine is rapid and complete through the oral mucosa. ST users have significantly lower salivary flow rates than age-, sex-, and race-matched non-user controls (10). In contrast, the concentration of IgA is significantly higher in ST whole saliva than control saliva. This increase has been attributed to the IgA2 subclass. Concomitant with the increase in IgA is a significant increase in J chain levels. However, the concentrations of SC, LF, and LY per microgram of total salivary protein are significantly lower in whole saliva of ST users than in that of controls. These results suggest an effect of ST on SEC. Therefore, to confirm the specific influence on SEC, the purpose of the present study was to examine the direct effect of ST, nicotine, and cotinine at

* Corresponding author. Mailing address: Department of Oral Biology, Indiana University, 1121 W. Michigan St., Indianapolis, IN 46202-5186. Phone: (317) 274-9949. Fax: (317) 278-1411. Electronic mail address: RGREGORY@IUSD.IUPUI.EDU.

concentrations normally found in ST users on synthesis and secretion of SC, LF, and LY by SEC in cell culture.

MATERIALS AND METHODS

Preparation of ST components. An aqueous extract of ST was prepared by emulsification of ST (moist snuff; University of Kentucky Tobacco and Health Research Institute, Lexington, Ky.) in sterile saline (10% [wt/vol]) and incubation at 37°C for 30 min. Particulate matter was removed by centrifugation at 10,000 × *g* for 10 min. The supernatant was filter sterilized through a 0.45-μm-pore-size cellulose acetate membrane filter (Corning Glass Works, Corning, N.Y.) and frozen in aliquots at −80°C until used. The ST extract contained approximately 0.6 to 3.4% (dry weight) nicotine (27). Pure nicotine and cotinine were obtained from Sigma Chemical Co., St. Louis, Mo.

Epithelial cell cultures. HT-29 human colon adenocarcinoma epithelial cells (ATCC HTB 38; obtained from American Type Culture Collection, Rockville, Md.) were used to establish the effects of the ST aqueous extract, nicotine, and cotinine on SEC synthesis and secretion of SC, LF, and LY. This cell line has been reported to produce SC in vitro (23) and also produced LF and LY. Because human recombinant interleukin-4 (rIL-4) and gamma interferon (rIFN-γ) have been shown to synergistically enhance (up to 40-fold) the expression of SC in HT-29 cells (23), experiments were conducted to address any protective effects of these cytokines on ST alterations of SEC metabolism. Cells were incubated in RPMI 1640 medium with fetal bovine serum (Intergen Co., Purchase, N.Y.) with and without human rIL-4 (10-U/ml final concentration; Genzyme, Boston, Mass.) and human rIFN-γ (10-U/ml final concentration; Genzyme) at 10^6/ml in 12-well flat-bottom sterile culture plates (1 ml per well; Corning) in the presence or absence of ST (0.001 to 10% final concentration of frozen ST aqueous extract stock), nicotine (0 to 1 mg/ml), and cotinine (0 to 5 mg/ml) for 5 or 7 days at 37°C in 5% CO_2. The approximate ST, nicotine, and cotinine concentrations selected were based on those of a previous report (7) and were similar to values typically reported for saliva of ST users (13). Cell culture supernatants (CS) were used to estimate levels of secreted SEC products and were removed and clarified by centrifugation (5,000 × *g*, 10 min). The cells were removed from the flasks by trypsinization and centrifugation (5,000 × *g*, 10 min). Cell lysates (CL) were used to estimate levels of synthesized SEC products and were prepared by sonication of the cells for 10 s, and the CL and CS were assayed for SC, LF, and LY by enzyme-linked immunosorbent assay (ELISA). CL were examined because many cell lines do not actively secrete surface-associated biomolecules. Furthermore, SC is present on the surface of HT-29 cells and is not entirely secreted. However, HT-29 cells have been reported to secrete SC when incubated with IL-4 or IFN-γ (23).

Determination of cell numbers, viability, and metabolic activity. Total cell numbers were established by measurement of the number of living and dead cells by microscopic counting, and cell viability was determined by trypan blue dye exclusion. Cell metabolic activity was assessed with a cellular metabolic activity assay kit (Promega Corp., Madison, Wis.). Briefly, trypsinized cells from cultures incubated with and without ST, nicotine, or cotinine were washed free of growth medium and resuspended to 10^5/ml in saline, and 50 μl was placed into a 96-well microtiter plate (Flow Laboratories, Inc., McLean, Va.). Thiazolyl blue (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MTT; 15 μl) was added to each well, and the plate was incubated for 4 h at 37°C in 5% CO_2. Solubilization solution (100 μl) was added to solubilize the cells, and the plate was incubated for an additional 4 h. The A_{540} and A_{650} of the wells were measured, and metabolic activity data were recorded as $A_{540-650}$.

Determination of SC, LF, and LY levels by ELISA. Polystyrene microtiter plates (enzyme immunoassay, Linbro, Flow) were coated (100 μl per well) with the CL or CS (diluted 1:10 in 0.1 M carbonate-bicarbonate buffer [pH 9.6]) and incubated at 37°C for 3 h. Coated plates were washed three times in Tween-saline (0.9% NaCl containing 0.05% Tween 20) to remove unbound antigen. Free sites on the plates were blocked by reaction with 200 μl of a solution containing 10 μg of globulin-free human serum albumin (Sigma) per ml in carbonate-bicarbonate buffer for 1 h at 25°C. The plates were washed three times with Tween-saline and incubated for 3 h at 37°C with 100 μl of horseradish peroxidase-labeled rabbit or goat anti-human SC-, LF-, or LY-specific reagent (1:1,000 [Nordic Immunological Laboratories, Tilburg, The Netherlands]). After three washes with Tween-saline, 0.5 M citrate buffer (pH 5.0) containing 0.0002% H_2O_2 and 0.5 mg of orthophenylenediamine dihydrochloride (Sigma) per ml was added (100 μl per well). Color development was monitored for 30 min, and the reaction was stopped with 2 N H_2SO_4 (100 μl per well). The amount of color which developed was measured at A_{490} in the microtiter plate with a Thermomax microplate spectrophotometer (Molecular Devices, Menlo Park, Calif.). The data were reduced by computing the means and standard errors of the mean of the absorbances of triplicate determinations per sample.

Statistical analysis. Each experiment was done between three and seven times, and representative experiments are shown. Variability (standard error of the mean) was generally <10% of the mean. Data are presented as mean percent change (cell number and metabolic activity and SC, LF, and LY levels) from the respective untreated control values without ST, nicotine, or cotinine. Differences among means were analyzed for statistical significance by the Mann-Whitney U test and Kruskal-Wallis one-way analysis of variance. Significant differences between groups were defined as $P \leq 0.05$.

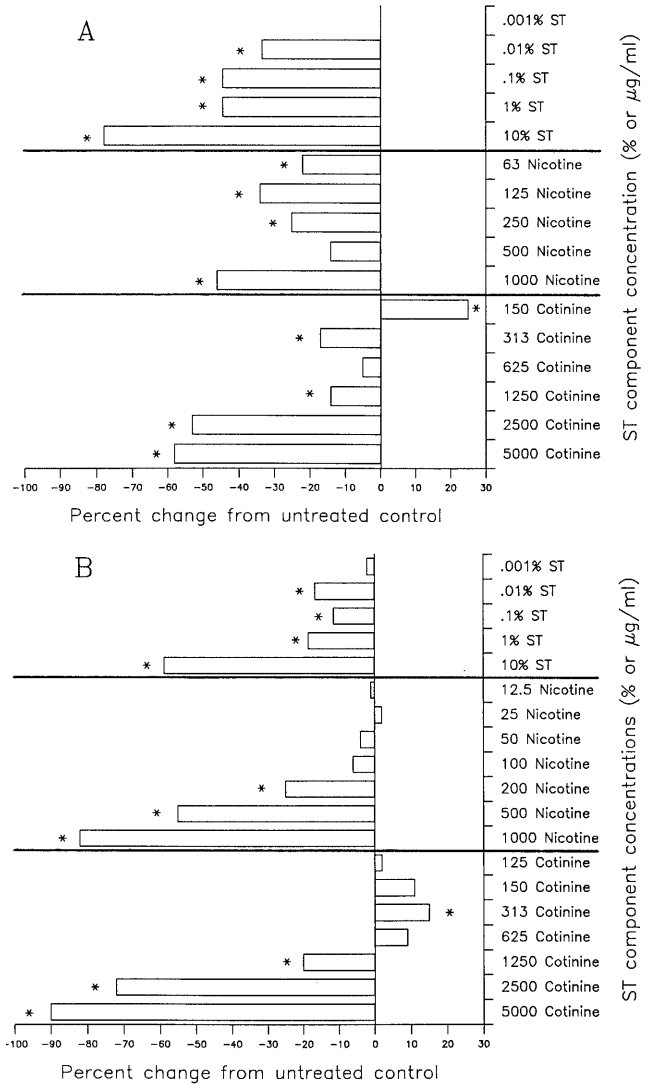

FIG. 1. Percent change from untreated controls of cell numbers (A) and MTT metabolic activity (B) of HT-29 epithelial cell cultures incubated for 7 days with 0.001 to 10% ST aqueous extract, 12.5 to 1,000 μg of nicotine per ml, and 125 to 5,000 μg of cotinine per ml. Positive and negative percent changes indicate enhancement and inhibition of the stated activity, respectively. Asterisks indicate significant differences ($P \leq 0.05$) from controls. Untreated controls did not contain ST extract, nicotine, or cotinine. The methods used are described in the text.

RESULTS

HT-29 epithelial cells incubated with ST extract demonstrated few significant differences in total cell numbers or metabolic activity from the untreated cells on day 5 (data not shown). However, by day 7, at the higher ST extract concentrations (greater than 0.01%), there were significant decreases ($P \leq 0.05$) in both cell numbers (Fig. 1A) and metabolic activity (Fig. 1B). Trypan blue dye exclusion experiments indicated cell viability ranged from 75 to 97% at all ST extract concentrations examined (data not shown). In similar experiments, the effect of nicotine on cell numbers at day 7 was significant only above 63 μg/ml. Furthermore, cellular metabolism assays provided similar information: 12.5 to 100 μg of nicotine per ml did not significantly affect metabolism, while 200-μg/ml to 1-mg/ml concentrations of nicotine significantly reduced cellular metabolism. The effects of cotinine on HT-29

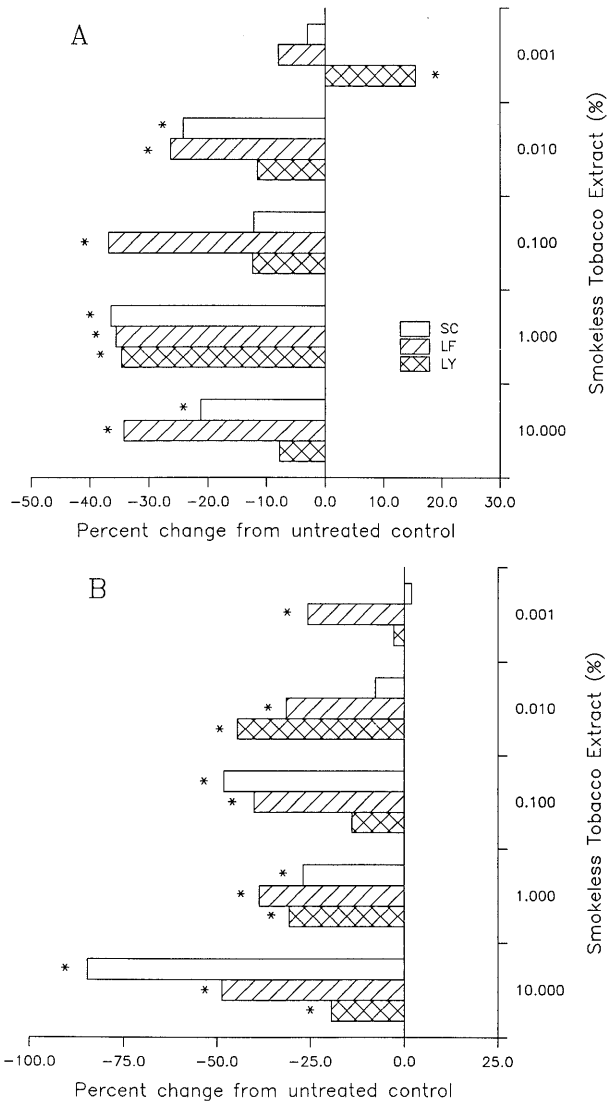

FIG. 2. Percent change from untreated controls of cell-associated (CL [A]) and secreted (CS [B]) SC, LF, and LY levels of HT-29 epithelial cell cultures incubated for 5 days with 0.001 to 10% ST aqueous extract. Positive and negative percent changes indicate enhancement and inhibition of the stated activity, respectively. Asterisks indicate significant differences ($P \leq 0.05$) from controls. Untreated controls did not contain ST extract.

cell viability and metabolism were not as great. Cotinine significantly reduced HT-29 epithelial cell numbers and cellular metabolism at concentrations greater than 313 μg/ml and 1.25 mg/ml, respectively.

Because of the apparent lack of ST toxicity at day 5, the effects of different concentrations of ST extract (0.001 to 10.0%) on HT-29 epithelial cell culture synthesis of cell-associated and secreted SC, LF, and LY after 5 days of incubation were analyzed (Fig. 2). HT-29 epithelial cells incubated with ST concentrations of 0.01% and higher concentrations had significantly decreased ($P \leq 0.05$) SC, LF, and LY levels in CL and CS. The 0.001% ST extract had no significant effect on SC and LF levels in CL and actually enhanced LY synthesis; however, secretion of LF was significantly depressed in CS at this ST concentration.

The addition of either rIL-4 or rIFN-γ diminished the negative effects that the 10% ST extract concentration had on HT-29 epithelial cell synthesis of SC, LF, and LY (Fig. 3). In a comparison between the ST-treated cells with the addition of rIL-4 or rIFN-γ and those without, the following differences were observed. (i) The addition of rIFN-γ significantly increased SC in CL. (ii) The addition of either rIL-4 or rIFN-γ significantly increased SC in CS. (iii) The addition of rIFN-γ significantly increased SC, LF, and LY in CL but only SC and LF in CS.

The effects of nicotine (12.5 μg/ml to 1 mg/ml) on HT-29 epithelial cell culture synthesis of cell-associated and secreted SC, LF, and LY were analyzed (Fig. 4). Briefly, lower concentrations of nicotine (12.5 to 100 μg/ml) had no significant inhibitory effects on SC, LF, or LY levels in CL. In contrast, concentrations of nicotine below 100 μg/ml enhanced LF and LY levels in CL. This may be associated with the increased cell number and metabolic activity observed with lower concentrations of cotinine and nicotine and may be associated with an increase in LY synthesis but not secretion. In general, concentrations of nicotine above 100 μg/ml depressed SC, LF, and LY levels in CL or CS the most. However, suppressive nicotine effects at lower concentrations were also noted. Nicotine significantly reduced the level of SC, LF, and LY in CS at concentrations between 12.5 and 100 μg/ml.

Cotinine significantly depressed SC levels in CL at concentrations greater than 1.25 mg/ml (Fig. 5). LF levels in CL were reduced only at and above 1.25 mg/ml and at 625 μg/ml in CS, while LY levels were significantly depressed at and above 1.25 mg/ml in CL and above 625 μg/ml in CS. SC levels were only depressed above 2.5 mg/ml in CL and at 5.0 mg/ml in CS.

DISCUSSION

The functional ability of the secretory immune system is the major line of defense against mucosal pathogens (for reviews, see references 9 and 20), and it is important to establish whether the use of ST affects this system. In this regard, Antoniades et al. (2) reported that ST reduced cytotoxic lysis of tumor cells by macrophages in vitro, indicating that cell-medi-

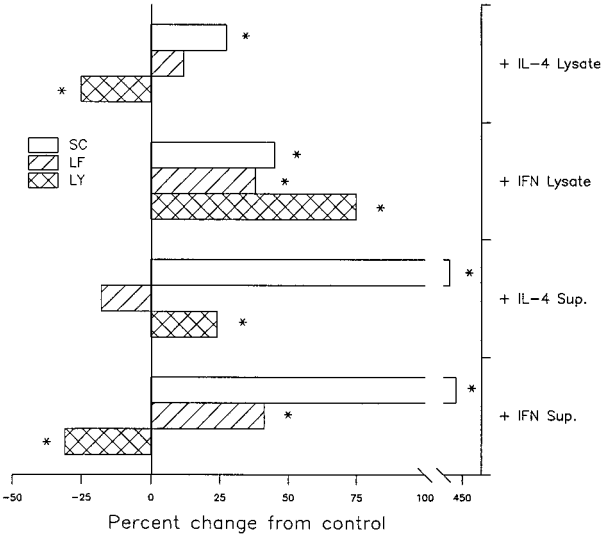

FIG. 3. Percent change from controls of cell-associated and secreted SC, LF, and LY levels of HT-29 epithelial cell lysates and culture supernatants (Sup.) incubated for 5 days with 10% ST aqueous extract and 10 U of rIL-4 or rIFN-γ per ml. Positive and negative percent changes indicate enhancement and inhibition of the stated activity, respectively. Asterisks indicate significant differences ($P \leq 0.05$) from controls. Controls contained 10% ST but no rIL-4 or rIFN-γ.

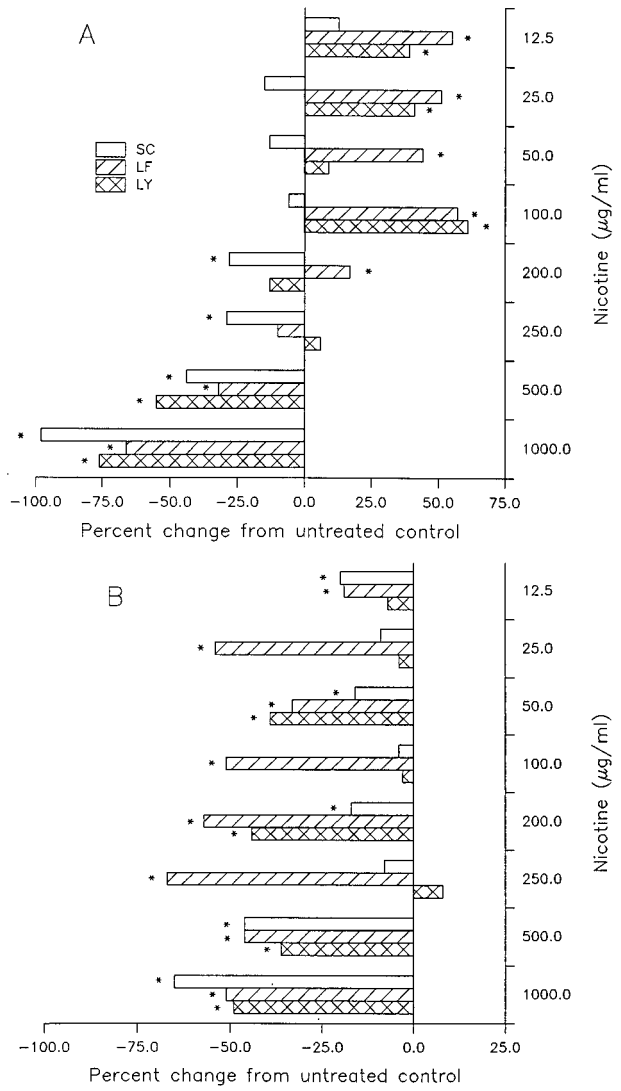

FIG. 4. Percent change from untreated controls of cell-associated (CL [A]) and secreted (CS [B]) SC, LF, and LY levels of HT-29 epithelial cell cultures incubated for 5 days with 12.5 to 1,000 μg of nicotine per ml. Positive and negative percent changes indicate enhancement and inhibition of the stated activity, respectively. Asterisks indicate significant differences ($P \leq 0.05$) from controls. Untreated controls did not contain nicotine.

ated immunity is affected. ST users had significantly lower salivary flow rates, which may be important (10). The mechanism associated with the lower salivary flow rates is likely due to the negative effect nicotine has on exocrine gland secretion, which has an initial stimulatory effect followed by an inhibition of saliva flow (26). Nicotine has a half-life of about 2 h after inhalation or parenteral administration and is metabolized into cotinine. Little is known regarding the biological effects of cotinine. The nicotine contents of ST products (either moist snuff or chewing tobacco) range from 0.6 to 3.4% (dry weight) or 6.1 to 24.5 mg of nicotine per g of tobacco (27). A single-use dose of tobacco contains approximately 14.5 to 133 mg of nicotine. ST users have approximately 1,500 μg and 10 to 50 ng of nicotine per ml of whole saliva and blood, respectively (13). Therefore, there is great potential for nicotine and cotinine to be present at or near SEC in ST users.

Other studies documented increases in oral pathology with decreased salivary flow rates (for review, see reference 24). Although ST users have copious amounts of saliva when chewing, our earlier data indicate significantly less salivary flow when not chewing tobacco (10). Even though ST users had significantly lower flow rates, they compensated with significantly higher concentrations of total whole salivary IgA which could be ascribed to the IgA2 subclass. Support for salivary fluid imbalance of ST users comes from a study of electrolytes and electrical potentials of buccal mucosal tissue treated with ST (28). This supports a possible effect on salivary flow and content differences between ST users and non-tobacco-using controls in the earlier study and suggests an effect of ST on the IgA2 subclass and on the packaging of soluble IgA with SC by SEC synthesis of SC, LF, and LY (10, 11). The Stensen's duct of the parotid salivary gland is longer and more convoluted than the more accessible submandibular, sublingual, and minor salivary glands. Therefore, it is likely that ST directly affects the

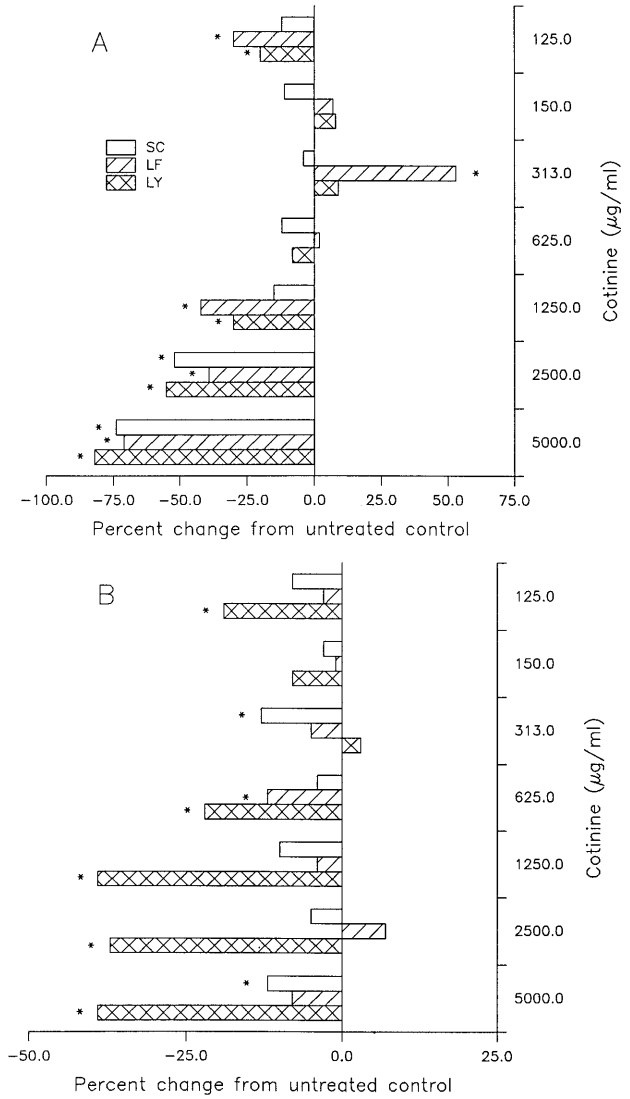

FIG. 5. Percent change from untreated controls of cell-associated (CL [A]) and secreted (CS [B]) SC, LF, and LY levels of HT-29 epithelial cell cultures incubated for 5 days with 125 to 5,000 μg of cotinine per ml. Positive and negative percent changes indicate enhancement and inhibition of the stated activity, respectively. Asterisks indicate significant differences ($P \leq 0.05$) from controls. Untreated controls did not contain cotinine.

SEC in the minor and/or submandibular and sublingual salivary glands, which are responsible for producing and packaging SC into IgA-J chain complexes synthesized in plasma cells (for reviews, see references 19 and 20). The much larger contribution of IgA from minor gland saliva than from parotid saliva (three- to fourfold higher [6]) and the relatively easier access of oral substances support the possibility of ST having an effect on minor salivary glands. No reports in the literature have documented such effects on SC packaging onto IgA by any substance.

Our previous antibody data indicate similar levels of salivary IgA and serum IgG and IgA binding antibody to *Streptococcus mutans*, the major etiological agent of human dental caries, by ELISA in ST users and controls (10). However, because of the lower salivary flow rates, the total contribution of salivary IgA antibody to *S. mutans* in the oral cavity of ST users was lower than that in controls. In general, saliva from non-tobacco-using controls significantly inhibited *S. mutans* acid production and glucosyltransferase functional enzyme activity to a greater extent than ST saliva. These functional antibody results supported our findings about ELISA binding antibody activity, in that control subjects had higher levels of anti-*S. mutans* antibody activity on a volume basis than ST users. However, this may not be important, because both ST and control groups had similar clinical (dental caries) parameters.

In the present study, HT-29 cells were cultured for 5 or 7 days at 37°C in 5% CO_2 in the presence of different concentrations (0.001 to 10%) of an aqueous extract of ST. ST significantly decreased ($P \leq 0.05$) the synthesis of SC, LF, and LY in CL and CS, particularly at the highest concentrations. However, low doses of nicotine increased synthesis of SC, LF, and LY, whereas high doses reduced SEC factor synthesis. In addition, lower concentrations of cotinine and nicotine increased cell number and metabolic activity (MTT) and may be associated with an increase in LY synthesis, but not secretion, suggesting some stimulatory effect. Synthesis of LF was significantly increased in cells treated with 313 μg of cotinine per ml, which may be associated with significant increases in cell metabolic activity at lower doses of cotinine. There may be different mechanisms involved in the synthesis and secretion of these important SEC factors. These results provide confirmation of a direct effect of ST components at concentrations normally found in ST users on SEC. SEC are physically exposed to the contents of the mouth (25), and it is easy to envision tobacco components contacting SEC directly or through the circulation and affecting metabolic pathways. Because other investigators have demonstrated up-regulation of SC levels by SEC with rIL-4 and rIFN-γ (23), experiments were designed to establish if these cytokines could abrogate the effects of ST. The decrease in SC, LF, and LY synthesis was moderated by the addition of rIL-4 and rIFN-γ. IL-4 was previously shown to costimulate, with nicotine, peripheral blood mononuclear cells to produce both IgA and IgG (7). Our data suggest a direct negative effect of ST on SEC leading to decreased synthesis of SC, LF, and LY at concentrations determined to be at or below cytotoxic, as well as inhibitory activity at lower concentrations of nicotine and cotinine. In addition, rIL-4 and rIFN-γ appeared to protect epithelial cells from the effects of ST on synthesis of these important mucosal defense factors. Furthermore, nicotine significantly decreased ($P \leq 0.05$) the levels of SC, LF, and LY in CL and CS at the highest concentrations. Our studies also indicated significant decreases ($P \leq 0.05$) in SC, LF, and LY levels in both CS and CL of cells cultured with cotinine. Cell metabolic activity decreased only when cells were incubated with higher concentrations of nicotine and cotinine. Differential effects of ST, nicotine, or cotinine were seen between the CL and CS values, suggesting a stronger effect of ST than nicotine or cotinine on either synthesis or secretion of SC, LF, or LY. This may be due to unknown tobacco components. Further studies are needed to determine the mechanisms of ST activity on SEC.

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