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A clinical, histomorphological and histochemical study on snuff-induced lesions of varying severity

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The oral lesions in 50 habitual snuff-dippers were graded on a four-point scale. The patients' tobacco and drinking habits were studied by means of a questionnaire. From each patient a biopsy was taken for histomorphological and histochemical analysis. A correlation between snuff habits and the clinical degrees was found, as well as between the snuff habits and certain superficial and deeply located cell changes. The incidence of keratinized lesions, sialadenitis and slight dysplasia (based on subjective evaluation under a light microscope) was higher than previously reported. Presence of dysplastic changes could not be predicted by means of the parameters which characterise the snuff habit or from the clinical grade. The histomorphological and histochemical results were interpreted as showing that the mucosa react to snuff inducing hyperplasia in the basal cell layers. In the surface layer indications of lethal damage were found. The overall stromal reaction to snuff was weak. However, the salivary glands and excretory ducts exhibited degenerative changes which were found to be more severe than the pathological changes in the surface epithelium.

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The clinical picture of snuff-dippers' lesions has been described by Pindborg & Renstrup (1963), Roed-Petersen & Pindborg (1973) and Axéll et al. (1976). These authors also presented the uniform histopathological picture of snuff-dippers' lesions in Scandinavia. Thus, increased epithelial thickness was observed in 80-100% of the cases. The surface layers of the epithelium were found to consist of vacuolated cells. Increased epithelial thickness and presence of vacuolated surface layers seemed to be positively correlated to the clinical severity of the lesions (Axéll et al. 1976).

In different parts of the world snuff has different constituents. Thus, the quality and content of tobacco and chemical components may vary widely as well as the manufacturing process (Roed-Petersen & Pindborg 1973). In Scandinavia, wet snuff, which is highly alkaline (pH 8-9), is used almost exclusively. Correlation between the histological appearance and the brand of snuff used has been reported (Pindborg & Poulsen 1962, Archard & Tarpley 1972, Axéll et al. 1976).

The daily exposure to snuff among snuff-dippers has been calculated to be 16 h by Roed-Petersen & Pindborg (1973) and 7 h by

Axéll et al. (1976). The latter authors estimated the consumption per day to be around 14 g. No correlation between the time of exposure or the quantities of snuff consumed and the clinical or histological appearance of the lesions has been reported.

It might be expected that differences in snuff habits would be reflected in different tissue reactions. The aim of this investigation was to study the clinical, histomorphological and histochemical characteristics of oral lesions, induced by exposure to snuff.

Material and methods

The material of the present study consisted of 50 male patients age $\bar{x} = 41.3 \pm 17.6$ (\pm SD), range 15–84 years, all habitual snuff-dippers.

Clinical methods

The clinical appearance of the snuff-induced lesions was graded in accordance with photographic standards and criteria given by Axéll et al. (1976).

- Degree 1 "A superficial lesion with a color similar to the surrounding mucosa with slight wrinkling and no obvious thickening"
- Degree 2 "A superficial whitish or yellowish lesion with wrinkling and no obvious thickening"
- Degree 3 "A whitish-yellowish to brown wrinkled lesion with intervening furrows of normal mucosal color and obvious thickening"
- Degree 4 "A marked white-yellowish to brown and heavily wrinkled lesion with intervening deep and reddened furrows and/or heavy thickening"

The snuff habits of the patients as well as

smoking and drinking habits were elicited by means of questionnaires as follows:

1. Number of years with the snuff habit . . . a
2. Daily exposure to snuff in hours . . . b
3. Daily consumption of snuff in grams . . . c
4. Total exposure $TE = a \times b \times c$
5. Brand of snuff used
6. Exact site for the placing of the quid
7. Smoking, chewing and drinking habits
8. Subjective symptoms

Histomorphological methods

From each patient a biopsy was taken for histomorphological and histochemical analyses. The tissue specimens, randomly enclosing minor salivary glands, comprised 10×5 mm of the upper vestibular mucosa and submucosa down to the underlying muscle tissue. Both peripheral (clinically unchanged) and central parts of the lesions were included in the biopsies. The specimens were immediately placed in ice-cold Histocon (Histo-Lab, Gothenburg, Sweden). The biopsies were frozen in isopentane chilled to about -140°C with liquid nitrogen (Heyden et al. 1972). Tissue sections ($8 \mu\text{m}$) were cut in a cryostat at -20°C . Cold microtome sections were fixed in 4% neutral buffered formalin and stained with haematoxylin and eosin and the van Gieson technique. These tissue sections formed a morphological reference for unfixed sections subjected to enzyme histochemistry.

Histochemical methods

a. Chemical component histochemistry

Cold microtome sections were fixed as above and stained for polysaccharides with the McManus PAS technique. Lipids were detected with the acid haematein method and the OTAN-technique (Adams 1965).

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Glucoseaminoglycans (GAGs) were stained with Alcian blue 8 GS pH 0.5 and 2.5.

b. Enzyme histochemistry

Unfixed cold microtome sections were incubated for the histochemical demonstration of glucose-6-phosphate-dehydrogenase (G-6-PD) and NADPH-diaphorase activities (Chayen et al. 1973). In order to disclose any metabolic atypia (aberrant G-6-PD-activity), tests were performed according to methods described by Heyden (1974, 1979). Acid phosphatase activity (azo-dye method, Burstone 1958) and leucylaminopeptidase activity (LAD; Nachlas et al. 1960) were also recorded.

The usual controls of possibly interfering non-enzymic staining reactions were performed (Chayen et al. 1973). Intrasection comparisons were made between clinically unchanged areas in the periphery of the lesions and central, pathological changes.

Statistical methods

In order to test mean differences of certain parameters in the different clinical degrees, one-way analysis of variance was performed.

If significant differences were found, multiple comparisons of the different means were carried out using the method of Scheffé (1959), significance level 5%.

Results

Clinical evaluations

The distribution of the lesions between the different clinical degrees is shown in Table 1. The ages and snuff habits of the patients are summarized in the same table. The four clinical degrees showed significant differences with regard to the parameters presented in the table. Younger patients were usually found in clinical degrees 1, 2 and 3, while a significant predominance of older patients was noted in degree 4. The mean number of years with the snuff-dipping habit was 20.2 ± 16.8 ($\bar{x} \pm SD$). The patients in the fourth clinical degree had been snuff-dippers significantly longer than the rest of the patients. On average, the patients kept the quid for 8.5 ± 4.9 h. As shown in Table 1, the patients in degrees 3 and 4 dipped approximately twice as long per day as the patients in degrees 1 and 2. The daily exposure to snuff in degree 4 was significantly longer than in degree 1. The differences be-

Table 1.
Degree of clinical change in relation to age and snuff habits.

Clinical degree	No	Age in years (\bar{x})	Number of years with the habit (\bar{x})	Daily exposure in hours (\bar{x})	Daily consumption in grams (\bar{x})	Total exposure (\bar{x})
1	10	32.1	9.2	5.2	6.8	167.0
2	9	27.3	9.2	6.5	15.2	1158.0
3	11	32.4	13.6	10.1	17.9	2910.3
4	20	57.2	34.7	10.6	22.3	12436.6
	50	41.3	20.2	8.5	17.1	4710.5
$\pm S D$		± 17.6	± 16.8	± 4.9	± 11.9	± 11843.3
Range		15-84	1-60	0.3-20	4-50	2.4-54175

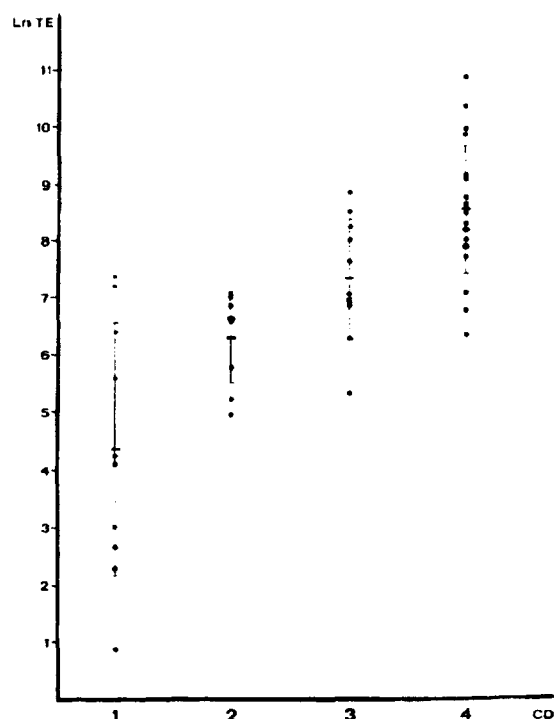


Table 2.
Graph showing the relationship between the clinical degree (CD) and total exposure (TE) to snuff ($n = 50$, $= \bar{x} \pm SD$).

tween the clinical groups concerning daily consumption of snuff was small. Only between degree 1 and degree 4 were significant differences found. When the total exposure (TE) was compared between the 4 clinical groups significant differences were found.

The relationship between TE and clinical degree is presented in Table 2.

The snuff-dippers used 8 different brands of snuff. However, 74% of the patients used 1 of 3 brands. Some patients changed brands quite frequently but a majority stated that they used only 1 or 2. More than half of the patients (68%) were social drinkers. Half of these patients were smokers as well.

Of those who did not drink any alcohol, 8% were smokers and snuff-dippers and 24%

used snuff only. No significant differences with regard to clinical grading and histological appearances could be found either between patients with multiple habits (snuff, smoking and drinking) and those who only used snuff or between patients who used different brands of snuff, and those who used one brand only.

Histomorphology

In general, the snuff-induced oral lesions were characterized by evenly distributed, slight (76%) to moderate (18%) parakeratinization. Vacuolated cells were noted in the superficial cell layers of the epithelium (76%) (Fig. 1) as well as varying degrees of stromal inflammation (80%). An increased epithelial thickness

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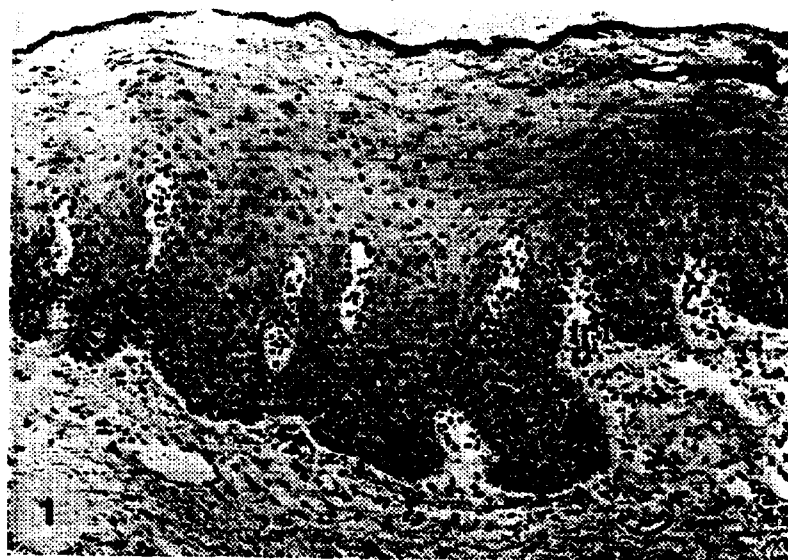


Fig. 1. Snuff-induced vacuolated cells in the superficial cell layers of the epithelium. Clinical degree 3. H&E $\times 125$.

Fig. 2. Slight epithelial dysplasia (including nuclear polymorphism and hyperchromasia in a weakly inflamed lesion of degree 2. Vacuolated cells are visible in upper parts of the spinous cell layer (S). H&E $\times 200$.

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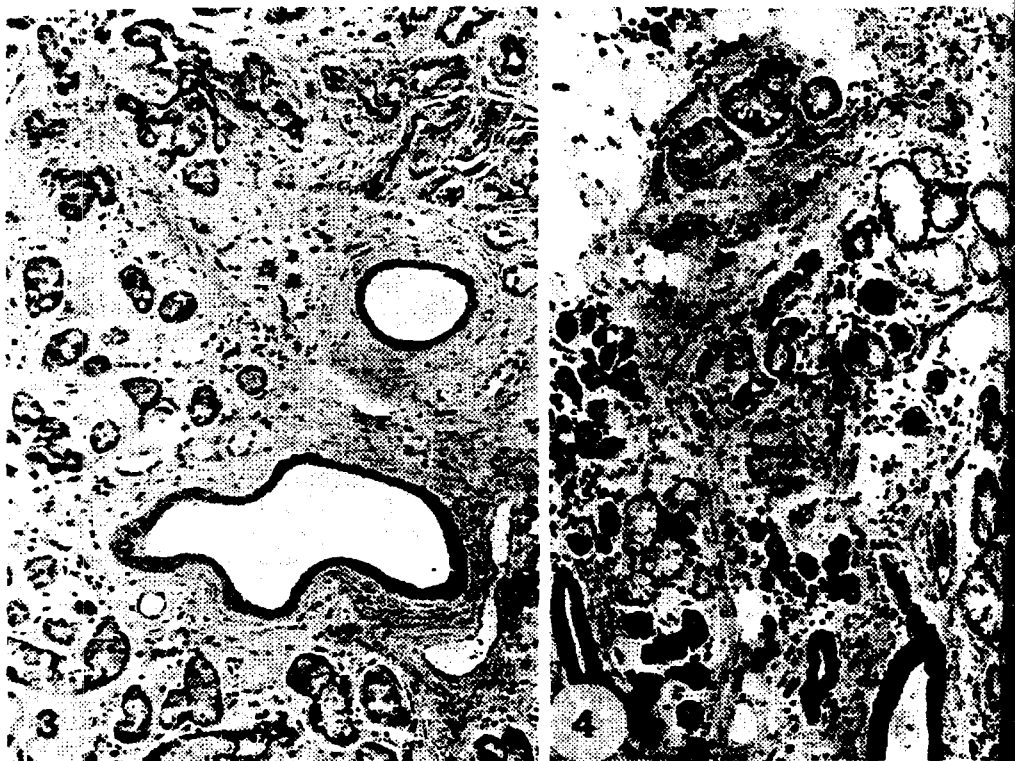


Fig. 3. Acinic cell degeneration with stromal hyalinization and dilated excretory ducts in a salivary gland in a lesion of clinical degree 4. H&E $\times 200$.

Fig. 4. Slight sialadenitis and partial degeneration of the parenchyma of a salivary gland. Clinical degree 2. H&E $\times 200$.

was seen in most of the specimens (94%). Atrophic and ulcerated lesions were rare (8%). Slight dysplastic changes (Fig. 2) were detected in the epithelium in lesions with varying degrees of inflammatory reaction (18%). For definition of dysplasia, see WHO Collaborating Centre for Oral Precancerous Lesions, 1978.

Lymphocytes and plasma cells were the predominating inflammatory cells. Significant

numbers of eosinophilic or neutrophilic granulocytes were not recorded.

In 2% of the non-inflamed lesions amorphous, weakly eosinophilic regions were found in the connective tissue papillae.

When the four different clinical degrees were compared concerning the severity of the above tissue changes, it was found that degree 4 usually demonstrated more accentuated changes. Vacuolization of the epithelial cells

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Table 3.
Dysplasia in relation to age and snuff habits.

Histological grading	No	Age in years (x)	Number of years with the habit (x)	Daily exposure in hours (x)	Daily consumption in grams (x)	Total exposure (x)
Dysplasia	9	44.7	23.9	8.3	17.0	4272.7
No dysplasia	41	40.6	19.5	8.6	17.1	4806.6
	50	41.3	20.2	8.5	17.1	4710.5
\pm s D		\pm 17.6	\pm 16.8	\pm 4.9	\pm 11.9	\pm 11043.5
Range		15-84	1-60	0.3-20	4-50	2.4-54175

(24%) as well as moderate parakeratinization (14%), was most prominent in degree 4 lesions.

Degree 4 lesions also included an increased number of mitotic figures, oedema and slight to moderate inflammation compared to the 3 other degrees. These tissue changes were also related to an increase in TE. It should be emphasized that lesions with slight epithelial dysplasia were found in all four clinical degrees and no significant correlation was found between any parameter presented in Table 3 and dysplasia. However, patients in the group with dysplasia had been snuff-dippers longer than the others. No case of moderate or severe dysplasia was recorded.

Tissue specimens from 74% of the patients included salivary glands. Sialadenitis and degenerative changes were registered in 42% of these. Fifty-two per cent of the lesions clinically belonging to degrees 3 and 4 demonstrated sialadenitis, while in degrees 1 and 2 this was found in only 19% of the biopsies including gland structures. Acinic cell degeneration was more prominent in lesions belonging to degrees 3 and 4 (Fig. 3) than in degrees 1 and 2 (Fig. 4).

Chemical component histochemistry

By using the PAS technique, a very unequal

distribution of polysaccharides could be demonstrated in the snuff-induced lesions. Single cells or groups of cells in the different strata of the epithelium displayed strongly positive staining reactions while the surrounding cells were totally negative (Fig. 5).

Lipid staining demonstrated diffuse distribution of unsaturated hydrophobic lipids in a granular form, both extra- and intracellularly, in the surface layers of the epithelium. Increased amounts of phospholipids in the cells of the more basal layers was noted in areas with high proliferative activity (Fig. 6).

A juxta-epithelial zone of sulphated mucopolysaccharides (GAGs) was demonstrated in lesional areas in some of the specimens. The above histochemical findings were made in all four clinical degrees. However, the positive lipid reactions seemed to be most prominent in degree 4.

Enzyme histochemistry

Recordings of G-6-PD activity as well as NADPH-diaphorase activity showed decreased enzymic reactions in the stratum granulosum. In the stratum basale a general increase of the oxidative enzyme activities was observed on intrasectional comparison between lesional and non-lesional areas in all four clinical degrees (Fig. 7).

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Fig. 5. Strong PAS positive reactions in single epithelial cells and cell regions in a lesion of clinical degree 3. $\times 200$.

Fig. 6. Black staining (B) of superficial epithelial cells indicating presence of unsaturated hydrophobic lipids. Strong brownish-red staining (arrows) indicating increased amounts of phospholipids in the basal cell layers of a lesion of degree 1. OTAN-staining. $\times 125$.



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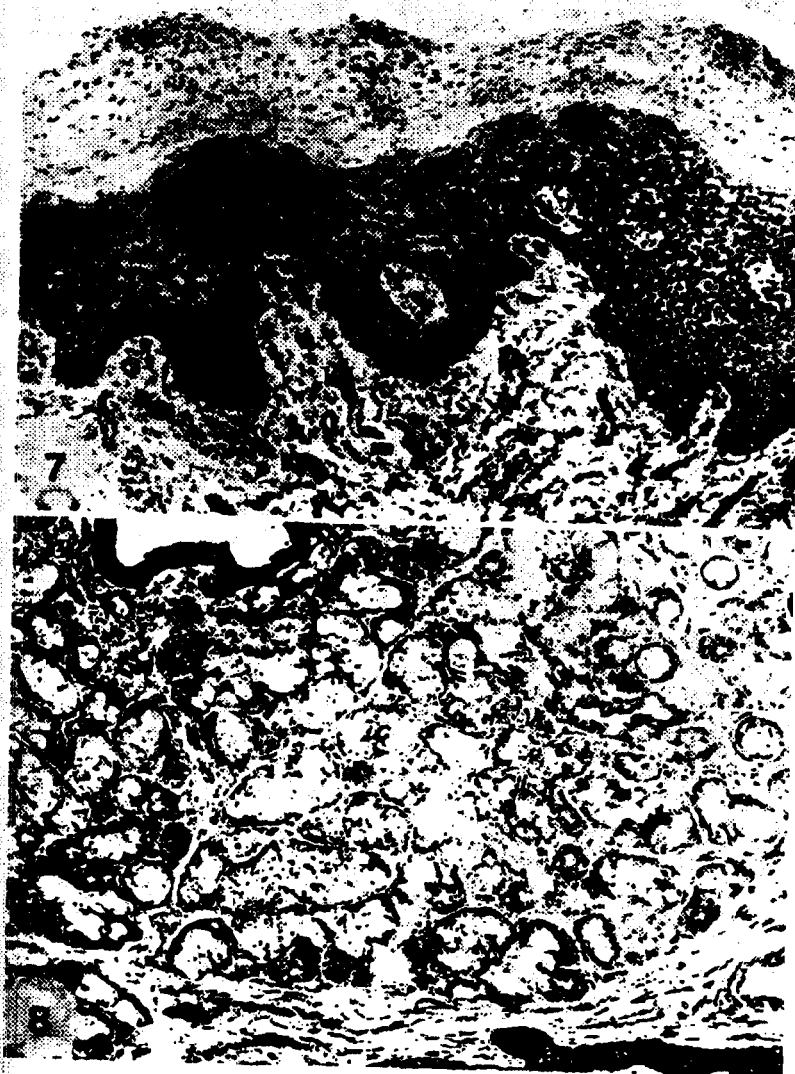


Fig. 7. Loss of NADPH-diaphorase activity in the superficial epithelial cell layers of a lesion of degree 2. Note the locally increased enzyme activity in the more basal cells in the same region. $\times 125$.

Fig. 8. Loss of G-6-PD activity in degenerating acinic cells in a salivary gland. Clinical degree 2. The dilated excretory ducts show strong enzyme activity. $\times 200$.

Acinic cells in salivary glands with sialadenitis and degenerative changes revealed weak oxidative enzyme activities (Fig. 8). In some dilated excretory ducts single epithelial cells or cell clusters stained for aberrant G-6-PD activity, while adjacent epithelial cells were almost unstained. These were the only signs of metabolic atypia recorded in available specimens. The oral epithelium was consistently negative in this respect. Normal distribution and activity levels of LAP were noted in the connective tissue. A slight increase in acid phosphatase reactions was observed in the basal cell layer of the oral epithelium. Both these enzyme activities were slightly increased parallel with increased degrees of inflammation.

Discussion

The patients in this investigation deviated in some respects from other studies. The patients were more evenly distributed within the groups (classified according to the clinical severity of the lesions) as compared to other studies (Axéll et al. 1976). Furthermore, the mean age of the patients was somewhat lower, the age range greater, and the patients had been snuff-dippers for a shorter time in comparison with other studies (Roed-Petersen & Pindborg 1973, Axéll et al. 1976). We also found that the snuff-dippers kept the quid fewer hours per day than stated by Roed-Petersen & Pindborg (1973) but 2 h longer than reported by Axéll et al. (1976). Finally, the daily consumption of snuff was greater in this study compared to estimates made by the above authors.

This investigation has demonstrated a higher incidence of keratinized lesions, sialadenitis, and biopsies with slight dysplasia, than reported earlier. Slight dysplastic changes were observed in all clinical stages of the lesions. In previous prospective reports

from Scandinavia only one case of snuff-associated dysplasia and one of snuff-induced squamous cell carcinoma have been described (Roed-Petersen & Pindborg 1973). Axéll et al. (1976) described changes characterized as "disquiet" epithelium in some specimens. Similar reactions to snuff had been reported earlier by van Wyk (1965).

The slight dysplasia observed in our study does not necessarily mean precancerous changes. As stated by the WHO Collaborating Centre for Oral Precancerous Lesions (1978), slight dysplasia may be found in non-precancerous lesions as well. However, in a retrospective study, Axéll et al. (1978) have demonstrated a clear correlation between snuff-dipping and oral cancer in Sweden, a fact suggested earlier in many other countries (for review, see Axéll & Mörnstad 1974). Thus, there is a documented inconsistency between prospective and retrospective investigations which remains to be further evaluated.

The present histomorphological and histochemical results support the interpretation that when oral mucosa is exposed to snuff, it reacts to the chemical injury with sublethal cell damage in deeper parts of the epithelium, inducing epithelial hyperplasia. The presence of hydrophobic unsaturated lipid in the surface layers of the epithelium might be due to cell degeneration (lethal damage). However, lipids might also be derived from the snuff quid. Non-enzymic staining reactions in the same areas, observed during recordings of oxidative enzyme activities, also indicate presence of lipids and sulphhydryl groups that might reduce the tetrazolium salts in the incubating media and thereby increase the staining reactions.

The regional presence of a juxta-epithelial band of sulphated mucopolysaccharides (GAGs) might be a stromal reaction to the external damage. The largely normal distribution and activity levels of LAP indicated that the stromal reactions to snuff were com-

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parably weak. The slight increase in acid phosphatase reactions observed in the basal cell layers of the epithelium might be involved in the local hyperplastic tendency of the epithelium.

An important observation in this investigation was that the salivary glands seemed to be subjected to more severe damage than the oral epithelium. The degrees of sialadenitis varied. Variations in degenerative changes in the salivary glands may be due to differences in snuff-dipping habits and brands of snuff. However, they may also reflect biological variations.

The most marked degenerative changes in the gland tissues were seen in the lesions clinically assigned to degrees 3 and 4.

Loss of local gland functions may lead to a decreased production of saliva and hence a decreased protection of the epithelium against snuff and other exogenous factors. The dilated excretory ducts also showed histochemical signs of reduced oxidative enzyme activities, indicating severe damage. The presence of aberrant G-6-PD-activity in some epithelial cells in such ducts remains to be explained.

Degenerative changes in salivary glands have been reported by other investigators (Roed-Petersen & Pindborg 1973, Axéll et al. 1976), but Archard & Tarpley (1972) found no evidence of such changes.

This investigation confirms earlier observations that the severity of the clinical picture is correlated to the superficial histopathological changes in snuff-induced lesions (Axéll et al. 1976). However, we also found a previously unreported association between the clinical degree and deeply located cell changes such as mitotic figures, moderate inflammation, oedema and sialadenitis. In this study the total exposure to snuff (TE) has been shown to be related to the clinical degree but not to presence of dysplasia. Thus, marked histomorphological changes, such as dysplasia, could not be pre-

dicted by using the suggested clinical degrees or by calculating the TE or any parameter characterizing the snuff habits presented in this study. This supports the hypothesis that there are other (endogenous and/or exogenous) factors involved in the development of severe snuff-induced lesions.

In conclusion, histomorphological and histochemical evidence was obtained in this investigation that snuff-dipping caused lesions reflecting alternating lethal and sublethal damage to, above all, the oral epithelium. Attention should, however, be directed towards the probably irreversible changes observed in the salivary glands and their excretory ducts.

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