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STUDIES IN ORAL LEUKOPLAKIAS

XI. HISTOPATHOLOGY OF LEUKOPLAKIAS IN INDIANS CHEWING "PAN" WITH TOBACCO

by

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In a pilot study by *Pindborg et al.* (1964), it was concluded that various tobacco-habits seem to cause different responses in the oral epithelium of leukoplakic lesions and also that the cheek mucosa seems to react differently from the labial mucosa. To complicate matters further, it became apparent that with the same oral habit in the same oral region the epithelium may exhibit either atrophy or hyperplasia. The purpose of the present study was to explore the features associated with epithelial atrophy in contrast to those associated with hyperplasia. In order to minimize the number of variables, the material was limited to leukoplakias from the cheek mucosa and to male patients whose oral habits were confined to the chewing of tobacco in a "pan". The study showed that despite these restrictions the epithelium again

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responded either by atrophy or by hyperplasia, and furthermore that all other quantitative aspects were likewise characterized by opposing extremes of behavior.

MATERIAL AND METHODS

Biopsies of the cheek were obtained in Bombay from 16 male patients with leukoplakias and from 6 healthy young males. The term "leukoplakia" did not carry any histological connotation. Leukoplakia was defined as a well-demarcated, elevated white patch of 5 mm or more in diameter that could not be scraped off and could not be attributed to the presence of other disease. A detailed statement of his oral habits was obtained from every individual. All patients chewed tobacco included in a "pan". [Pan is the Hindi word for a preparation of betel leaf, betel nut raw or cured, slaked lime and catachu.] The bolus formed by chewing the preparation was either spat out, swallowed or kept in the mouth for hours.

After measurements of epithelial thickness had been made on histologic sections, the patients were divided into two groups. The first group included the 8 patients who had supplied the thinnest epithelia: "thin group"; the second group included the remaining 8 patients: "thick group". The 6 young males who acted as controls had never chewed or smoked tobacco. The average age of the patients was 40 years, that of the controls was 30 years.

The biopsies were obtained under local anesthesia, usually by a 5 mm punch instrument, from areas within the lesions that had a uniform appearance. The tissue was fixed in 10 per cent formalin, embedded in paraffin and serially cut at 5 μ. Sections were stained with hematoxylin and eosin, the periodic acid-Schiff (PAS) reagent (*Hotchkiss,* 1948), Azure-B (*Flax, et al.,* 1952), Feulgen's stain (*Pearse,* 1960) and Mallory's triple connective-tissue stain as modified by *Weidenreich* (1926). Sections stained with H and E were used for general morphological and for quantitative studies. The PAS technique controlled by diastase digestion was used to study the distribution of glycogen. Azure-B was used to study nucleoli and cytoplasmic basophilia indicative of RNA; Feulgen stain was used to study nuclear changes; Mallory's stain, to study keratin and connective tissue.

Quantitative determinations were made of thickness of epithelium and of the keratin layer; spacing and height of connective tissue papillae; relative length of basal layer; number of mitoses per 100 μ of epithelium. The measurements were taken on drawings of projected sections under a magnification of about 40×. Average thickness was obtained by planimetry. Biopsies containing epithelium of markedly heterogeneous thickness were divided and treated as two separate biopsy specimens from the same patient. The thicker of the two regions was used for classifying the patient. Such subdivisions were made in four biopsies from the eight patients of the thin group and in two biopsies from the eight patients of the thick group, resulting in 12 regions in the "thin group" and ten regions in the "thick group". Mitotic frequency was determined by counting under oil immersion the number of mitoses in sections of known length. The number of dividing cells per biopsy varied from one to 349 and averaged 78. Care was taken to examine all material available when the mitotic frequency seemed exceptionally high or low. The stretches of epithelium examined varied from 0.3 to 3.0 cm of actual length, averaging 1.3.

Semiquantitative estimates of the severity of inflammation were made by microscopic inspection. Density and depth of the inflammatory infiltrate were taken into account. The specimens were ranked in the order of increasing inflammation and a rating of 10 given to the one with severest inflammation. Then the other specimens were given grades in proportion to the first one. With suitable modifications, similar methods were used for other estimates, e. g. of the density of inflammatory cells in the Malpighian layer of the epithelium, the degree of intraepithelial edema, the proportions of para- and orthokeratin in the stratum corneum. Independent observers could always reach agreement about the rank order, though not necessarily about the exact numerical rating given each specimen.

RESULTS

I. Quantitative differences between leukoplakias and controls

1. Averages (Table I)

Epithelial thickness in the 6 controls averaged 647 μ; in 22

Table I: *Average differences between controls and leukoplakias*

	Average		Standard Deviation*		Number of Observations	
	Controls	Leukoplakias	Controls	Leukoplakias	Controls	Leukoplakias
Epithelial thickness, μ	647	450	107.5	282.4	6	22
Distance between papillae, μ	234	256	43.3	103.1	6	22
Height of papillae, in % of epithelial thickness	36	37	4.9	11.6	6	22
Ratio of length of basal layer and epithelial surface	3.0	2.7	.26	1.9	6	22
Number of mitoses per 100 μ length of basal layer	.096	.178	.0184	.107	6	19
Number of mitoses per 100 μ length of surface	.289	.581	.018	.573	6	19

* Calculated as the square root of $\frac{\text{sum of the squared differences}}{N-1}$

leukoplakias the average was 450 μ. The average distance between connective tissue papillae was 234 μ in the controls and 256 μ in the leukoplakias. The average height of the papillae was 36 % of the epithelial thickness in the controls and 37 in the leukoplakias. The length of the basal layer averaged 3.0 times the length of the epithelial surface in the controls, and 2.7 times this length in the leukoplakias. The number of dividing cells per 100 μ length of surface averaged .29 in the controls, and .58 in the leukoplakias. Per 100 μ length of basal layer, dividing cells averaged .096 in the controls and .178 in the leukoplakias. Except for greater epithelial thickness in the controls and for higher frequency of cell division in the leukoplakias, the differences between the averages were slight. Due to the great variability of the leukoplakias no difference was statistically significant.

2. Variability (Table II)

In every quantitative determination, the range of the leukoplakias extended far below as well as above the control range. The one percent confidence level, defining the range that contains 99 % of the normal population, was adopted for evaluating the

Table II: *Variability of controls and leukoplakias*

	Range	Probable Limits of Normal*		Number of Leukoplakias Outside of Normal Range	
	Control	Leukoplakias		Below	Above
Epithelial thickness, μ	494—774	107—1153	370—924	12	1
Distance between papillae, μ	180—305	104—489	123—346	1	5
Height of papillae in % of epithelial thickness	30—43	17—56	23—49	4	4
Ratio of length of basal layer and epithelial surface	2.56—3.31	1.26—9.86	2.3—3.7	12	5
Number of mitoses per 100 μ length of basal layer	.074—.124	.038—.356	.048—.143	2	13
Number of mitoses per 100 μ length of surface	.231—.381	0.05—1.96	.244—.334	6	11

* Calculated as the average $\pm$ 2.578 times the standard deviation of the six controls. (R. A. Fisher, Statistical Methods for Research Workers, 1944, p. 77).

limits of the "normal". Epithelial thickness in the 6 controls varied from 494 to 774 μ; in 22 leukoplakias the range was 107 to 1153 μ. 13 leukoplakias were outside the normal range; 12 were thinner and one thicker. The average distance between connective tissue papillae in the controls varied from 180 to 305 μ, in the leukoplakias from 104 to 489 μ. One was below and five were above the normal range. The height of papillae in the controls was between 30 and 43 % of the total epithelial thickness; in the leukoplakias the range was from 17 to 56 %. In four, the papillae were shorter and in four taller than normal. The length of the basal layer in the controls was from 2.56 to 3.31 times the length of the epithelial surface; in the leukoplakias the range was 1.26 to 9.86. In 12, the length was below and in 5 above the normal range. The number of mitoses per 100 μ surface length ranged from .231 to .381 in the controls. The range in the leukoplakias was .05 to 1.96. In 6, the activity was below normal limits and in 11 above. Per 100 μ length of the basal layer, mitoses in the controls ranged from .074 to .124, and in the leukoplakias from .038 to .356. The number was below normal in 2 and above normal in 13.

Table III. *Averages and coefficients of variation of measurements in leukoplakias with thin thick epithelium*

	Average			Coefficient of variation (%)		
	Thin Group	Thick Group	Control	Thin Group	Thick Group	Control
Epithelial thickness, μ	263	674	647	27.9	41.2	16.6
Distance between papillae, μ	276	232	234	39	42	18.5
Height of papillae, μ	102	230	230	49	48	17
Height of papillae, % of epithelial thickness	37.9	35.1	35.9	32	33	14
Ratio of lengths of basal layer and epithelial surface	1.97	3.58	2.98	43	68	8.7
Number of mitoses per 100 μ length of basal layer	.163	.175	.096	60.7	65.1	20.8
Number of mitoses per 100 μ length of surface	.396	.785	.289	97.7	87.4	19.0

II. Differences between thin and thick leukoplakias (Table III)

1. Epithelial thickness and configuration

Thickness (Graph 1). The epithelia of the *"thin" group* of leukoplakias averaged 263 μ and exhibited differing degrees of atrophy. The *"thick" group* averaged 674 μ and consisted of mildly atrophic specimens, specimens of normal thickness, and hyperplastic specimens. Epithelial thickness in each group of leukoplakias was about twice as variable as in the controls.

Spacing of papillae. Similar average spacing of the papillae was found in thin and thick leukoplakias. However, both groups contained specimens with wider as well as specimens with closer spacing of papillae than did the control group.

Height of papillae. The absolute heights of the papillae differed, but the relative heights had similar group averages. Again both groups of leukoplakias were represented below as well as above the control range. The heights of papillae in leukoplakias were even more varied than the spacing.

Interrelations of epithelial thickness and configuration of the connective tissue=epithelial junction:

Distance between papillae and epithelial thickness were not

related. Extremely small distances occurred in epithelia from the entire range of thicknesses. Extremely wide spacing of papillae occurred in thin epithelia as well as in epithelia of intermediary thickness. The absence of correlation was especially striking in the thin group.

Height of papillae and epithelial thickness were likewise unrelated. Again the absence of correlation was especially impressive in the thin group, in which short and tall papillae were randomly scattered over the whole range of thicknesses.

Relative length of basal layer and epithelial thickness. The relative length of the basal layer averaged almost twice as much in the thick group as in the thin group. The control average was intermediary. At the extremes, relative length of basal layer and thickness were somewhat correlated (Graph 1). In the epithelia of intermediary thickness, the length of the basal layer was between 2.5 and 3.5 times the length of the surface, and the values were distributed randomly in epithelia of 250 to 800 μ thickness.

There was a striking contrast between the constancy of the relative length of the basal layer in the six control specimens and its extreme variability in both groups of leukoplakias. In the controls this was the least variable of all measurements, highest and lowest value differing by less than 30 %. In the leukoplakias, the variability was 5 to 8 times as great. The values in both groups, especially in the thick one, were scattered far below and above the control range. A representative control specimen and the extremes of epithelial configuration in the leukoplakias are illustrated in Figures 1—7.

2. Mitotic activity

Mitotic rate per 100 μ length of basal layer. Thin and thick group of leukoplakias averaged quite similar numbers of dividing cells per 100 μ length of basal layer. The averages were almost twice as high as the average of the controls, but the rates in both groups were three or more times as variable.

Mitotic rate per 100 μ length of surface. Per 100 μ surface length, the frequency of dividing cells was twice as high in the thick group of leukoplakias as in the thin group. Both groups

averaged higher mitotic rates than the controls, the thin group by about a third, the thick group more than twice as high.

Thickness and level of mitotic activity (Graph 2). The mitotic rate was feebly related to the thickness of the epithelium. Few dividing cells were found in the extremely atrophic specimens, but at thicknesses between 270 and 400 μ, the mitotic rates of the thin group were randomly distributed. The thick group did not contain specimens with extremely low mitotic rates and showed slightly better correlation of thickness and rate than the thin group.

Mitotic activity and relative length of basal layer (Graph 3). In the controls, mitotic rate as well as the relative length of the basal layer were rather constant, but in both groups of leukoplakias extremely variable. In the latter groups, a good correlation was present between the mitotic frequency per 100 μ surface length and the relative length of the basal layer. Mitotic activity was extremely low when the length of the basal layer was below 1.6 times the surface length of the epithelium. A sharp rise occurred when the relative length of the basal layer was greater, with mitotic rates rising about twice as fast as the length of the basal layer. Thin and thick group fitted the same trend line. Only one region, from the thick group, showed a marked deviation. The behavior of both groups of leukoplakias differed strikingly from that of the control group: with a given relative length of basal layer, specimens from both the thin and the thick group showed much higher mitotic activities than did control specimens with the same relative length of basal layer.

III. Histopathologic observations

1. Findings related to keratinization

Incidence of keratinization. None of the six controls but all leukoplakias showed either frank keratinization or distinct keratinizing changes. Frank keratinization was seen in the specimens of the thin group and in the two thinnest specimens of the thick group of leukoplakias (Figs. 2, 3, 4, 6, 8). In these specimens the bulk of the epithelial cells were of the keratinizing type and a layer of keratin was present at most of the surface, despite in-

flammation in the underlying connective tissue and despite secondary signs of inflammation in the epithelium. In the six thickest leukoplakias, keratinizing changes were present but did not amount to frank keratinization (Figs. 7, 10, 11). The lower third of the cellular layer presented a weak version of the habitus of keratinizing cells; the outer two-thirds showed traits of non-keratinized epithelium; the surface layer consisted of cells which appeared indistinctly keratinized.

Cell layers in keratinized specimens. Basal and spinous cells of the fully keratinized specimens showed the features seen in normally keratinized human oral epithelium. The basal cells were of large size, the spinous cells were of angular shape and had prominent prickles and multiple prominent nucleoli. The cytoplasm was markedly basophilic and contained no glycogen. These traits were sometimes of abnormal prominence and sometimes only feebly expressed. The former was the case in regions covered by a thick stratum corneum. In such regions the exaggerated keratinizing traits of the lower layers were often followed by a markedly early decline in nucleolar prominence and pale staining of the chromatin with the Feulgen technique. Prominence or feeble expression of keratinizing traits were not related to the degree of inflammation in the underlying connective tissue. Features characteristic of keratinizing epithelium did, however, tend to be weaker in regions with secondary *intra-epithelial* signs of inflammation. Foci of marked secondary changes due to inflammation were often associated with small non-keratinized islands (Fig. 5). In these islands nucleoli and cytoplasmic basophilia faded early, the cells from the middle on out contained small amounts of glycogen, and a clearly identifiable stratum corneum was absent.

A granular layer was present. Its thickness varied from a small fraction to more than a third of the total epithelial width. As in normal oral epithelium, the presence of a granular layer was limited to orthokeratotic parts of specimens. Its absence from parakeratotic parts was strikingly exemplified in some specimens (Fig. 8) with abrupt juxtaposition of ortho- and parakeratin: the elaboration of keratohyaline granules ceased as abruptly as did the disintegration of the nuclei. Both width of the granular layer and the granule content of the cells were excessive (Figs. 3, 8) in

specimens with a thick stratum corneum consisting of pure orthokeratin.

Cell layer in semikeratinized leukoplakias. A peculiar mixture of the traits of keratinized and unkeratinized regions of oral mucosa or of traits intermediary between both characterized the six semi-keratinized leukoplakias (Fig. 7). In the lower third of the cell layer angular shape of the cells, wide intercellular spaces, marked basophilia, prominence of nucleoli, and scarcity of glycogen approached the picture in normal keratinizing epithelium rather than that in the unkeratinized epithelium of the control. Basophilia in the controls was maintained for at most five rows of cells, then decreased rapidly, but was of constant intensity in the entire lowest third of the epithelium in the leukoplakias. Nucleoli were more prominent than in the controls, though less than in the frankly keratinized specimens. Glycogen, which in the controls appeared as early as the third cell row and not later than in the tenth, was altogether absent from the basal one-third of the epithelium. In the middle third the cells took on traits characteristic for non-keratinizing regions. Angular shape and intercellular spaces disappeared, giving way to large empty cells typical for formalin-fixed non-keratinized epithelium. Cytoplasmic basophilia dwindled and glycogen appeared, though not reaching its maximal concentration before the outer half of the epithelium or even later. Granules of the size of KHG's and of a comparable degree of basophilia were present in the six control specimens in a few to a considerable number of rows of cells, ending at or near the surface of the epithelium (Fig. 9). The cells containing granules were not flattened. Unlike the irregular KHG of keratinized epithelium (Figs. 3, 10), the granules of control specimens were of precise spherical shape. In shape, size and basophilia, they were the exact counterpart of granules in exfoliated cells from unkeratinized regions of the oral cavity (unpublished observations). They resembled the granules illustrated by *von Bülow* (1966) in electron micrographs of human cheek epithelium and by *Osmanski* (1966) in that of mouse cheek. They occupied a minute fraction of the cytoplasm; the number per cell varied from 2—3 to 15—20. In semikeratinized leukoplakias, a granular layer comparable to that in the keratinized specimens was absent. Instead they contained spherical basophilic granules

identical with those in the controls (Fig. 10). Cells containing such granules were more numerous than in the controls, but the granules still occupied only a small fraction of the cytoplasm.

Keratin layer in keratinized and semikeratinized specimens. In the controls the surface layer was unkeratinized but contained occasional flattened parakeratinized cells with eosinophilic cytoplasm and a flattened pyknotic nucleus. Such cells formed small clusters over papillas of the connective tissue or discontinuous rows at the surface. In the keratinized group of leukoplakias a distinct keratin layer covered the bulk of the epithelial surface. Except in small islands with marked secondary signs of inflammation, ortho- and parakeratinized cells were flattened, though to a variable degree. In the semikeratinized leukoplakias, keratinizing changes in most of the cells in the superficial rows were distinct enough to mark a layer of measurable thickness (Fig. 7), but otherwise of differing degrees of resemblance to true keratinization. The nuclei were usually pyknotic, but the cells rarely truly flattened. A layer of keratin was absent in the thickest specimen, but clusters of parakeratinized cells were present.

Thickness of keratin. Although composed of much flatter cell residues, the keratin layer in the thin group averaged nearly the same thickness as in the thick group: 56 against 60 μ. The relative thickness of the keratin layer varied widely, but was far greater in the thin group. In only one specimen of the thick group but half those of the thin group, the keratin layer measured more than 20 % of the total thickness (Graph 4).

Type of keratin. No specimen was wholly ortho- or wholly parakeratinized. Orthokeratotic, parakeratotic, and unkeratinized regions were combined in various ways. Often one was juxtaposed to the next, sometimes quite abruptly (Fig. 8). Sometimes parakeratinized cells occupied the deeper half of the keratin layer, but orthokeratin or non-keratinized cells occupied the peripheral layer. Where encrustations of pan leaves had burrowed into the surface, a layer of parakeratin lined the pit and constituted a keratinized island in a non-keratinized surface (Fig. 11).

The proportion of orthokeratin increased with increasing relative thickness of the stratum corneum (Graph 5). When the latter measured more than 30 % of the total epithelial thickness, the bulk of it consisted of orthokeratin. Since the thickest stratum

corneum was found in the most atrophic specimens, the highest proportions of orthokeratin were found in these specimens. Orthokeratin amounted to more than 60 % of the stratum corneum in the thinnest six specimens; 26 % in the next six, 16 % in the next group and zero in the thickest group. In the first three groups, parakeratin replaced orthokeratin; in the thickest group, nonkeratinized epithelium was the dominant type (Graph 6).

It was possible in six specimens to compare the thickness of the keratin layer and the mitotic activity (Graph 7). Almost no dividing cells were found when the stratum corneum was more than 30 % of the total epithelial thickness. With decreasing proportion, a marked fairly regular increase in mitotic activity was seen.

2. *Specific histopathology and inflammation*

No specific pathologic changes were widespread. Ulcers were seen in one specimen each from thin and thick group. Both were severely inflamed. In one, numerous fungi were present on the surface. The epithelium in the vicinity of the ulcer was extremely thin but well keratinized. Two more specimens, both from the thin group, showed candida albicans at the surface. In both, the relative length of the basal layer and the mitotic rate were exceptionally high. Both came from patients with long years of chewing and high daily chewing times.

Encrustations of pan leaves on the epithelial surface were frequently seen, especially in the thick group. The residues were often located in more or less shallow depressions and occasionally in veritable pits. In the case illustrated in Figure 11, enough tissue was available to demonstrate for the region underlying and surrounding the pit that the epithelium was parakeratotic whereas elsewhere it was unkeratinized; that the connective tissue papillae were more closely spaced and reached closer to the surface of the epithelium; and that the mitotic rate was about twice as high as in the remainder of the specimen. These changes seemed to be typical for lesions caused by burrowing of the pan into the epithelial surface. Inflammation was somewhat more marked in the connective tissue underlying such lesions than elsewhere in the specimen. Nonetheless they were associated with an increased

degree of keratinization, which was demonstrable both at the surface and in the Malpighian layer.

Cellular atypia was not encountered. Mitoses, even when greatly increased in frequency, appeared normal. In the thinnest specimens, occasional prematurely keratinized cells were seen. More frequent, especially in the most atrophic regions, was premature loss of Feulgen stainability of the chromatin. Nuclear pyknosis was infrequent.

Inflammation

Lamina propria. No leukoplakia was wholly uninflamed. Inflammation was far more severe in the thin group (Graph 8). Only one specimen of this group, but eight of the thick, had the two mildest ratings of severity. Ten regions of the thin group were rated grade 4 or higher, but none of the thick group rated higher than 3. The thin group averaged a rating of 5.1, the thick group of 2.0. The thin group tended to widespread severe inflammation, whereas the thick group had a tendency to mild general inflammation but focal flare-ups, e. g. in regions underlying encrustations of pan.

Intra-epithelial intercellular edema was in no instance very marked and often absent. Figure 12 illustrates the maximal degree; to this the rating 4 was given. Like underlying inflammation, intra-epithelial edema also was far more severe in the thin group. Most regions in this group rated 2 or 3; the majority in the thick group rated zero (Graph 9). The average rating of the thin group was 2.4; that of the thick group was 0.7. Intra-epithelial edema also tended to be widespread in the thin, but focal in the thick group.

Intra-epithelial inflammatory cells. Nearly every region of both groups contained some inflammatory cells in the deepest one-third of the cellular layer. In the middle one-third, six specimens of each group were free of migrating cells, and in the outer third all but two regions of each group had none. The ratings of density of migrating inflammatory cells had similar distributions (Graph 10) and identical averages in the thin and thick groups.

These similar distributions of density and the identical averages of migrating inflammatory cells were in striking contrast to the

marked differences in the degree of inflammation and of edema, both of which were far more severe in the thin group.

IV. Age and oral habits of patients in thin and thick groups

Age. The age distributions of the patients in the thin and thick groups were closely similar (Graph 11). The average age was 40 years in the thin group and 44 in the thick group, or 40, if the one old patient in the group is excluded.

Duration of chewing habit. The duration of the habit was slightly shorter in the thin than the thick group (Graph 12). In each group was one patient who dated the beginning of his habit no more than a year back, and four patients who had been chewing for more than 20 years. The thin group had no chewers of more than 24 years standing, the thick group had one who had chewed for 30 and one who had chewed for 33 years. The average duration in the thin group was 16 years and in the thick group 18 years.

Intensity of chewing habit. An estimate of the intensity of the habit was obtained by multiplying the number of pans chewed per day by the minutes of chewing per pan. Longer daily chewing times were by far more common in the thin group (Graph 13). Five of the eight patients of this group exceeded daily times of 80 minutes and two exceeded daily times of 105 minutes. In the thick group, no patient exceeded 75 minutes and four of eight chewed no more than 20 minutes. The average daily chewing time was 74 minutes in the thin group and 37 minutes in the thick group.

DISCUSSION

The purpose of this study was to develop a quantitative profile of a group of leukoplakias associated with a single, well defined oral habit. It was believed that a quantitative approach was best suited for comparisons between leukoplakias associated with different oral habits and arising in different patient populations. The findings in six controls were encouraging; for they exhibited unexpectedly narrow ranges of variation in all traits. This constancy made it possible to set up rather small ranges for the val-

ues to be expected in the normal population, against which deviations in pathological material could be evaluated.

It was found, however, that the outstanding characteristic of the present pathological material, 22 leukoplakias from 16 patients chewing tobacco in pan, was its extreme variability. Confirming pilot studies, the epithelium was found to be either atrophic or hyperplastic, with less than half the material in the range of normal thickness. Most other quantitative traits of the leukoplakias were similarly characterized by clustering above and below, but rarely within the control range. In view of this centrifugal tendency, these leukoplakias could not adequately be described by means of group averages and significant differences between controls and leukoplakias. A twofold quantitative approach was used instead. In the first place, quantitative determinations or estimates of relevant variables were made in each specimen, and the series of numerical values examined for presence or absence of statistical association with one another. As a result of their narrow ranges, the control values were usually found in a cluster somewhere near the center of the range of leukoplakic values. Thus a much larger control material would have to be examined for establishing correlations among biologic variables within normal buccal epithelium. But in the leukoplakias, some suggestive correlations between variables were noted, e. g. between the relative length of the basal layer and the mitotic rate. Secondly, the leukoplakias were divided into two groups according to the thickness of the epithelium, and the differences between the specimens and patients supplying the thin and thick group were studied. In comparing the present to other pathological entities, the tendency to extremes will similarly have to be kept in mind. Preliminary comments on some of the present findings which are deemed interesting in their own right are given below.

Configuration of the connective tissue epithelial junction:

Determination of spacing and height of the connective tissue papillae, which together determine the size of the basal layer relative to the size of the epithelial surface, may prove to be valuable for characterizing mucosal lesions.

In the control specimens, the coefficient of variation of the

relative length of the basal layer was only about half as great as the coefficients of variation of either spacing of papillae or height alone. It was the least variable of all measurements, suggesting that in the normal mucosa the size of the exchanging surface between epithelium and connective tissue is kept rather constant. The exact opposite was true in the leukoplakias. In both thin and thick groups, spacing and height of the papillae in the majority of specimens were within the normal range, but the relative size of the basal layer was twice as variable as either factor alone, and 18 of 22 specimens were outside the normal range. It seemed that deviation from normal of one factor was associated with deviation of the second one in the same direction, thus causing amplified changes in the relative size of the basal layer. It was clear that this behavior was important in the histopathology of the leukoplakias, since in both groups there was a positive correlation between the relative size of the basal layer and the mitotic activity. In the thin leukoplakias the relative length of the basal layer averaged less and in the thick group more than in the controls. For a given relative length, the mitotic activity in both thin and thick leukoplakias was higher than in the controls.

Mitotic activity:

Per unit length of basal layer, mitotic activity in both groups of leukoplakias averaged twice as much as in the controls. Per unit length of surface, it averaged 1.4 times as much in the thin group and 2.7 times as much in the thick group. It will be noted (1) that the turnover rate of the basal cells, in which the bulk of cell renewal occurs, was accelerated in both groups of leukoplakias, and (2), that atrophic and hyperplastic groups did not differ in the mitotic rate per unit length of basal layer. It should be kept in mind that the mitotic rate per unit surface determines the rate at which new cells accrue to a given region, either to add to the thickness of the epithelium or to be desquamated at the surface. It is interesting that in the six controls mitotic activity per unit surface was less variable than mitotic activity per unit of basal layer. For comparing the proliferative capacity of different materials, rate per unit of surface rather than rate per unit of basal layer is the relevant measure. So measured, the proliferative capacity of the hyperplastic group was twice as great as

that of the atrophic group. This was mediated by the greater relative length of the basal layer in this group.

The present findings indirectly confirmed *Renstrup's* (1963) findings of higher mitotic rates in parakeratinized than in orthokeratinized leukoplakias. Because of the irregular interspersing of ortho- and parakeratin (Fig. 4) in the present material, a direct comparison was impossible. However, the predominantly parakeratotic thick group had twice the rate of cell division per unit surface as the predominantly orthokeratotic thin group. In six leukoplakias, mitotic activities could be compared in a thinner and a thicker part of the same specimen. In three pairs the mitotic activity was lower in the thin part and in three pairs higher. This discrepancy was found related to the relative thickness and type of keratin in the two parts. In three pairs, the thin part had the thicker keratin layer, containing more orthokeratin, and it had the lower mitotic activity. In the other three pairs, the thin part had the thinner keratin layer, which contained more parakeratin. These were the specimens with higher mitotic activity in the thin part.

Frank keratinization:

Conversion to a keratinizing type is a well-known reaction of buccal mucosa to changed external or internal conditions. It was seen in frank form in the atrophic half of the present material. In spite of having a predominantly keratinized epithelium, this group was characterized by twice as severe a degree of underlying inflammation and of intra-epithelial edema as the thick group, in which frank keratinization had not occurred. The keratinized-inflamed group had similar distributions of age and standing of the chewing habit, but markedly longer daily exposure times to tobacco-in-pan.

Frank keratinization despite severe inflammation similarly persists in buccal lichen planus and in early submucous fibrosis, where marked inflammation may underlie a keratinized epithelium (*Pindborg et al.*, 1965). By contrast, strictly inverse relations between degrees of keratinization and of inflammation of oral mucosa have been noted in the clinically normal human gingiva and in the palate, i. e. in inherently keratinized regions (*Weiss et al.*, 1959), but also in keratinized oral cysts (*Pindborg*

et al., 1962 and 1963) and in keratinized buccal mucosa lining fibromas (*Gerson & Meyer*, 1963). The reason for these contrasting responses to inflammation is not clear. A possibly relevant finding of the present study was the lack of association of migrating inflammatory cells and severity of inflammation: specimens maintaining keratinization despite edema and underlying inflammation did not also have to contend with increased numbers of inflammatory cells in the epithelium.

It may be instructive to compare keratinization of buccal mucosa associated with fibromas of the cheek (*Gerson & Meyer*, 1963) and with chewing of tobacco in pan. In the latter instance one can single out a group of specimens, in which keratinization perhaps represents a protective adaptation, as exemplified by instances of very thin but well-keratinized epithelium in the vicinity of ulcers. In this group the epithelium had a virtually straight basal layer with nearly no dividing cells, but was covered by a thick smooth layer of orthokeratin, in which few encrustations of pan leaves were seen. One might suspect that such epithelium has turned into a non-renewing but friction-resistant and impermeable tissue. This is different from the purely friction-induced adaptation in the mucosa lining buccal fibromas. In the absence of inflammation, such mucosa also has a well-keratinized orthokeratotic epithelium, but one that shows marked elaboration of epithelial ridges and a higher mitotic frequency than the epithelium of origin, i. e. that responds to increased friction with keratinization plus an increased rate of cell production. Early stages in the response to chewing of tobacco-in-pan can perhaps be recognized in the vicinity of surface pits caused by burrowing encrustations in thick non-keratinized epithelium (Fig. 11). Such regions showed a layer of keratin lining the pit, but betrayed the permeability of the epithelium by the increased degree of underlying inflammation, the lengthening of the basal layer, and the increased mitotic rate.

Semikeratinization:

The hyperplastic specimens among the present leukoplakias were often difficult to classify as keratinized or unkeratinized. The lower third of the cellular layer showed typical features of keratinizing epithelium, while in the upper two thirds features of

nonkeratinizing mucosa were present. The cells of the "stratum corneum", except for being distinctly set off by their different staining, resembled compressed surface cells with pyknotic nuclei more than keratinized cell residues.

Since these specimens were associated with long years of chewing, it cannot be assumed that the mucosa was in transition from a nonkeratinized to a keratinized state. *Meyer & Gerson* (1964) suggested that differentiation of stratified lining epithelium into either the keratinizing or the unkeratinizing type is the consequence of the appropriate determination of the basal cells. Findings like the present ones make it likely that this concept is an oversimplification. Maintenance of a given pathway of epithelial differentiation may, in addition, require that the cells superficial to the basal layer must have an environment that is compatible with continuing maturation along the initially induced pathway. Exposure to one or another of the constituents of the pan package may create conditions which interfere with the continuation of keratinizing processes.

Effects of patients' age, duration and intensity of habit:

Younger age and shorter years of the chewing habit were slightly more common in the group of patients furnishing the atrophic than in the group furnishing the thick half of the present material. Epithelial hyperplasia was slightly more common in older age or longer-standing chewing habits. A much larger sample will be needed to assess the strength of these associations.

The strongest association observed was between atrophy and the intensity of the chewing habit. The length of time per day of chewing tobacco-in-pan was overwhelmingly correlated with epithelial atrophy. It will be of great interest to study the association between intensity of the oral habit and atrophy of the epithelium in other habits and other populations.

One striking exception from the positive relation between intensity of the chewing habit and epithelial atrophy was a patient with markedly atrophic epithelium, who had chewed for less than a year and reported the briefest daily exposure time. Such patients may deserve closer attention, e. g. with respect to the presence of submucous fibrosis.

SUMMARY

Quantitative determinations were made in a group of 22 leukoplakias of the cheek obtained in Bombay, India, from 16 male patients chewing tobacco in pan and in six control specimens. There were no marked differences between group averages, but leukoplakias and controls differed strikingly in the small variability of the controls and the great variability of the leukoplakias. In every quantitative trait, at least one third of the leukoplakias and usually the great majority had values below and above the probable range containing 99 % of controls.

Leukoplakias with atrophic and with thick epithelium averaged similar spacing and relative height of connective tissue papillae. In the thin group the length of the basal layer relative to that of the surface was one half that in the thick group. The number of mitoses per 100 μ basal layer was the same in both groups, but the number per 100 μ surface was twice as high in the thick group. Mitotic activity according to both measures averaged higher values in both groups of leukoplakias than in the controls. Mitotic activity of individual leukoplakias correlated poorly with epithelial thickness and well with the relative length of the basal layer.

All leukoplakias showed frank keratinization or semikeratinization. In every keratinized specimen, both para- and orthokeratin were present. Nearly no dividing cells were present in specimens with orthokeratin layers of 30 % or more of the total epithelial thickness. Semikeratinized specimens resembled keratinized specimens in the deeper cell layers, but non-keratinized epithelium in the outer part.

Keratinizing traits declined with marked intra-epithelial reaction to inflammation but persisted against all degrees of inflammation in the lamina propria. Inflammation and intra-epithelial edema were more than twice as marked in the atrophic group, but the density of migrating inflammatory cells was the same in both groups of leukoplakias. Leukoplakias of both groups came from patients of similar age and fairly similar duration of the chewing habit. However, the daily exposure time to tobacco in pan was, on the average, twice as long in the patients with atrophic epithelium.

RÉSUMÉ

ÉTUDES SUR LES LEUCOPLASIES BUCCALES

Des déterminations quantitatives ont été faites sur un ensemble de 22 cas de leucoplasies de la joue provenant de 16 patients de Bombay, Inde, du sexe masculin, mastiquant le tabac dans le bétel, et sur six prélèvements témoins. Il n'existait pas de différence marquée entre les moyennes des deux groupes, mais il existait une différence frappante dans les degrés de variabilité, les témoins présentant une variabilité faible, et les leucoplasies une grande variabilité. Pour chaque caractéristique quantitative, les valeurs trouvées pour au moins un tiers des leucoplasies, et même en général pour la plupart d'entre elles, étaient soit inférieures soit supérieures à l'intervalle probable comprenant 99 % des témoins.

La distance entre les papilles du tissu conjonctif ainsi que leur hauteur relative étaient en moyenne semblables dans les leucoplasies présentant un épithélium atrophique et dans celles qui présentaient un épithélium épais. La longueur de la couche basale par rapport à celle de la surface était, dans le groupe mince, la moitié de la longueur correspondante dans le groupe épais. Le nombre de mitoses par 100 μ de couche basale était le même dans les deux groupes, mais le nombre de mitoses par 100 μ de surface était deux fois plus élevé dans le groupe épais. L'activité mitotique trouvée lors de ces deux mesures était en moyenne plus élevée dans les deux groupes de leucoplasies que dans le groupe témoin. L'activité mitotique des leucoplasies prises individuellement présentait une corrélation faible avec l'épaisseur de l'épithélium et une forte corrélation avec la longueur relative de la couche basale.

Toutes les leucoplasies présentaient une kératinisation franche ou une semikératinisation. Dans tous les cas de kératinisation, on trouvait et de la parakératine et de l'orthokératine. Presque aucune cellule en voie de division n'était présente dans les cas où l'épaisseur des couches d'orthokératine étaient de 30 % de l'épaisseur totale de l'épithélium. Les cas de semikératinisation ressemblaient aux cas de kératinisation dans les couches profondes de cellules, mais, dans la partie externe, ils ressemblaient à de l'épithélium non kératinisé.

Les caractères de kératinisation diminuaient avec les nettes

réactions intra-épithéliales envers l'inflammation, mais ils persistaient quelque fût le degré d'inflammation dans la lamina propria. L'inflammation et l'oedème intra-épithélial étaient au moins deux fois plus marqués dans le groupe atrophique, mais la densité des cellules inflammatoires en migration était la même dans les deux groupes de leucoplasies. Les leucoplasies des deux groupes provenaient de patients d'âges similaires et ayant eu l'habitude de mastiquer le tabac pendant à peu près la même durée. Cependant la durée journalière de mastication du tabac dans le bétel était en moyenne deux fois plus longue chez les patients présentant un épithélium atrophique.

ZUSAMMENFASSUNG

STUDIEN ÜBER MUNDLEUKOPLAKIEN

Zweiundzwanzig Leukoplakien aus der Wangenschleimhaut von 16 Patienten und Kontrollmaterial von 6 Subjekten gleichen Alters wurden einer quantitativen Untersuchung unterzogen. Das Material stammte aus Bombay, und alle Patienten waren Gewohnheitskauer von tabakenthaltendem "Pan".

Im Durchschnitt zeigte das Patientenmaterial nur geringe quantitative Verschiedenheiten von den Kontrollen, zeichnete sich aber durch seine ausserordentliche Variabilität aus. Für weitaus mehr als die Hälfte der Leukoplakien ergaben sich in allen quantitativen Bestimmungen Werte oberhalb und unterhalb der wahrscheinlichen Streuungsbreite in einer normalen Bevölkerung.

Leukoplakien mit atrophischem und hyperplastischem Epithel ähnelten einander mit Bezug auf Abstand und relative Höhe der Bindegewebspapillen. In der atrophischen Gruppe war die Länge der Basalschicht im Vergleich zur Länge der Epitheloberfläche um die Hälfte kürzer als in der hyperplastischen. Auf die Länge der Basalschicht berechnet zeigten beide Gruppen die gleiche Mitosenhäufigkeit. Dagegen war die Zahl der Mitosen mit Bezug auf die Oberfläche in der atrophischen Gruppe nur halb so gross wie in der hyperplastischen. Beide Berechnungsarten ergaben im Patientenmaterial höhere Mitoseziffern als in den Kontrollen. Die Mitosenhäufigkeit zeigte wenig Beziehung zur Dicke des Epithels, war aber von der relativen Länge der Basalschicht eng abhängig.

Volle oder unvollständige Grade von Verhornung waren in allen

Leukoplakien feststellbar. Alle enthielten Abschnitte von Para- und von Orthokeratin. Zellteilungen fehlten, wenn die Dicke der Orthokeratinschicht sich auf mehr als ein Drittel der gesamten Epitheldicke belief. In Epithelien mit unvollständiger Verhornung glichen die tieferen Zelllagen den entsprechenden Lagen in verhornendem Epithel, die höheren dagegen den entsprechenden Zellen nicht verhornender Schleimhäute.

In Gewebsstückchen mit starker Entzündungsreaktion *im* Epithel waren nur geringe Anzeichen von Verhornung auffindbar. Anderseits aber behauptete sich der Verhornungsprozess gegen alle Grade von Entzündung im subepithelialen Bindegewebe. Die atrophische Gruppe zeichnete sich aus durch höhere Grade von intra-epithelialem Ödem und von sub-epithelialer Entzündung; die Zahl der intra-epithelialen Entzündungszellen war in beiden Gruppen die gleiche. Auch zeigten beide Gruppen die gleiche Streuung von Alter und Jahresdauer des Tabakskauens. Die tägliche Kauzeit dagegen war in der atrophischen Gruppe um ein doppeltes länger als in der hyperplastischen.

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