CYTOLOGY OF THE ORAL MUCOSA BY A FILTER IMPRINT TECHNIC*

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In 1940, Wolf (1) described a method of examining the surface of the skin by stripping off the most superficial horny layer by means of transparent adhesive tape. Pinkus (2) followed a similar procedure and described the use of Scotch-brand transparent adhesive tape to remove consecutive superficial layers of epidermis by applying a fresh tape for each layer of epithelial cells. This adhesive method is limited in application to the dry skin.

Many technics have been devised to collect superficial cells from the various regions of the moistened oral mucosa. Some of the more popular methods include use of various types of metal spatulas as described by Montgomery (3), Weinmann (4), King (5), and Ziskin (6); wooden blades as advocated by Miller (7, 8), Sandler (9, 10) and Stone (11); cotton-tipped applicators used by Peters (12, 13) and Silverman (14, 15); and edge of a glass slide scraped on the mucosa by Marberger (16). Scheman (17) prefers using the abrasive action of the tongue and teeth during the rinsing of the patient's mouth with a physiologic saline solution.

In such scraping and rubbing procedures, however, the number of cells collected varies considerably; morphologic cell characteristics may be distorted and there is a further loss of topographic relationship in the mixing of the cells during the cell collection. Thus a need was seen for the removal of superficial cells by direct contact with some type of adhesive material that would restrict removal to one or two layers of cells without distortion or damage to these cells. Other requirements are that the material be nontoxic to the mucosa, be relatively easy to manipulate and not interfere with suitable staining for microscopic viewing.

Since cellulose ester filters are used to recover neoplastic cells by filtration methods (18) and since blood cells can adhere to such a filter-paper surface (19, 20), the most promising device for meeting the above requirements was the Millipore filter.† This type of filter has certain characteristics: It does not interfere with staining qualities of the cells; retains the topographic cell relationship to one another; and finally it can be rendered transparent without appreciable distortion or loss of morphology of the cells.

The purpose of this study was to test the reliability of the filter imprints technic by examining the types of cells found on the surface of the normal oral epithelium with the degree of keratinization of the epithelium.

MATERIALS AND METHODS

The Millipore filter, type HA, 13 mm in diameter, white, with grid lines, was used for this study (Fig. 1). The grid squares proved desirable when differential counts and grouping of the cells were made from the filter imprint.

The dry filter was held with small fine-pointed forceps and placed directly on the mucosal area to be studied (Fig. 1); the forceps were removed momentarily; the filter pressed against the tissue with the fore-finger while wearing a rubber finger cot, in a slightly rolling manner to cover the filter. The filter was immediately removed with the forceps and promptly placed in 95% ethanol for fixation. The total time for the procedure from placement of the filter to the fixation required only 10 to 15 seconds.

A simple but effective staining rack was made by spot-welding paper clips at right angles to an .045″ stainless steel round wire. The paper clips were modified by having two opposing ends of equal length which exert a slight pressure sufficient to hold the filter and to prevent wrinkling. The wire was designed with a closed loop at one end and an open loop at the other, so that the staining rack holder can be squeezed slightly and easily placed in a standard staining dish (Fig. 2). The rack facilitated the attachment of the filters during staining, dehydration and clearing procedures.

During the study at least two consecutive filter imprints were routinely taken of a selected area of the oral mucosa. The first filter imprint was stained by the Papanicolaou method (21), slightly modified for this study (Table I), and the second imprint with Mallory's connective tissue stain (22), also slightly modified (Table II). The filter imprints were dehydrated in alcohols, cleared in xylol and permanently mounted on glass slides

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† Millipore Filter Corp., Bedford, Mass.

‡ Xylocaine, Astra Pharmaceutical Products, Inc., Worcester 6, Mass.

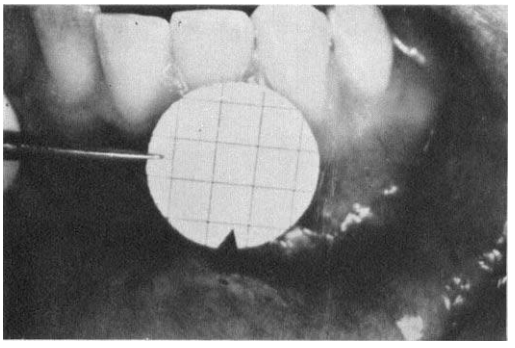


FIG. 1: A dry Millipore filter, with a small "V" notch cut out for orientation of the area, is being held by fine-pointed forceps during the application for a sample that will include portions of the free gingiva, attached gingiva and remaining alveolar mucosa.

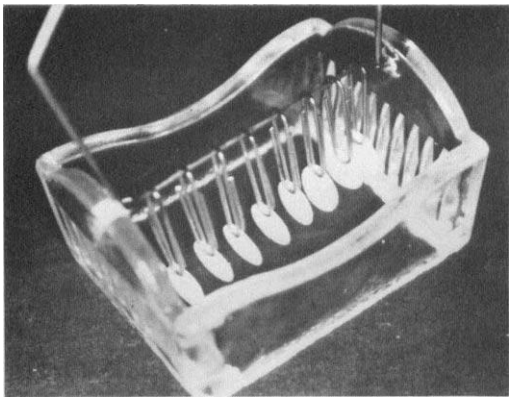


FIG. 2. The complete staining rack assembly with the filter discs attached is shown placed in a rectangular glass immersion tray. The modified paper clips exert a slight pressure to hold the filters and prevent their wrinkling.

with H.S.R. (Harelco Synthetic Resin) in 60% toluene. A range of 400 to 1,000 cells from each imprint were differentially counted from random areas of the filter for statistical evaluation. The differential count included precornified, cornified and keratinized types according to the Papanicolaou's classification for the cytoplasmic staining reaction of the cells.

In addition to the above described procedure, the filter may be orientated to the area by making a small "V" notch in one direction (Fig. 1) before applying the filter to the mucosal surface. For example, if the filter is placed in such a manner as to include free gingiva, attached gingiva and a portion of the alveolar mucosa (Fig. 1), it can provide additional regional information.

Since this was a different method of obtaining superficial epithelial cells of the oral mucosa, it was essential that a comparison be made between the normal cytology of the filter imprint and the normal histology of the same area in order to establish the reliability of the imprint technic. Thus, immediately after the last imprint of the same selected area, the surrounding tissue was infiltrated with 2% xylocaine‡ and a biopsy specimen was taken and fixed in 10% neutral formalin. Representative paraffin sections were prepared for hematoxylin and eosin (H & E) and Mallory's stain (Table II). The stained sections were dehydrated, cleared in the usual manner and mounted on glass slides using D.P.X. mountant.

In the present study 49 complete samples employing both filter imprints and biopsy specimens were taken from 41 human subjects. The tissues examined were clinically normal areas of mucous membrane from the buccal, at the occlusal line of the first permanent molar region; lower labial, near the midline; lower lingual mandibular alveolar mucosa, at the cuspid and premolar areas; free and attached gingiva, at the mandibular and maxillary premolar areas; crest of the alveolar edentulous ridge, at the posterior areas; and hard palate, at various areas.

The study was conducted on a random sample ranging in age from 13 to 62 years. There were 30 males and 11 females, and 33 Caucasian and 8 non-Caucasian individuals.

FINDINGS

Mallory's stain did not differentiate clearly between non-cornified and cornified cells in the filter imprint technic. Its use in the cytologic portion of this study was therefore discontinued.

On the other hand, the filter imprints stained with the slightly modified Papanicolaou stain provided good color differentiation and cytologic detail. This stain made it possible to differentiate between precornified (blue to green cytoplasm), cornified (pink to red cytoplasm) and keratinized (yellow to orange cytoplasm) cells according to Papanicolaou's criteria. (Fig. 3, 4, 5) The pyknotic nuclei predominantly seen in vaginal smears (23) were low in number in this study of the oral mucosa. They varied according to the following (based on the total arithmetic mean of each filter imprint cell type): (1) precornified cells from 0.8% to 3.3% (mean of 1.6%); (2) cornified cells from 3.3% to 6.1% (mean of 4.9%); and (3) keratinized cells from 0.9% to 8.1% (mean of 4.3%).

The filter imprints provided cytologic detail that was sufficient to distinguish between the various stages of maturation of superficial epithelial cells. Findings differed depending upon the area involved, *e.g.*, the buccal mucosa which

TABLE I

Papanicolaou staining procedure

Solution or Stain	Standard	Modified
Fixative	Alcohol-Ether	95% Ethanol
Hydrate in 80%, 70%, 50% alcohols and distilled water	6 dips* each	2 minutes each
Harris's hematoxylin (without acetic acid, diluted to 50% with an equal amount of distilled water)	6 minutes	2.5 minutes
Rinse in distilled water	Rinse	None
Wash in running tap water	None	5 minutes
Aqueous solution of HCl, six drops	0.25%	0.5%
Wash in running tap water	6 minutes	5 minutes
Dehydrate in distilled water, 50%, 70%, 80% and 95% alcohols	6 dips* each	2 minutes each
OG-6 Stain†	1.5 minutes	4 minutes
Rinse in 95% alcohol, two changes	6 dips* each	2 minutes each
EA-50 Stain†	1.5 minutes	4 minutes
Rinse in 95% alcohol, three changes	6 dips* each	2 minutes each
Dehydrate and clear by running through absolute alcohol, mixture of absolute alcohol and xylol equal parts (1:1)	Approx. 6 dips* each	None
Dehydrate and clear by running through absolute alcohol, 1-propanol, mixture of 1-propanol and xylol equal parts (1:1)	None	3 minutes each
Xylol, two changes	6 dips* each	10 minutes each
Permount, gum damar, Canada balsam or any other satisfactory neutral medium	Mountant	None
H.S.R.‡ in 60% toluene (xylene may be substituted)	None	Mountant

* Preece, A.: A Manual For Histologic Techniques. 1st Ed., Little, Brown and Co., Boston, 1959, p. 87–88.

† Prepared and marketed by Ortho Pharmaceutical Corp., Raritan, N. J.

‡ Hartman-Leddon Co., Philadelphia, Pa.

varies from non-keratotic to incomplete parakeratotic exhibited greater propensity of cell exfoliation than did the keratotic palatal mucosa. In addition to epithelial cells, particularly in the free gingival areas, there were at times polymorphonuclear leukocytes, micro-organisms and other oral debris (Fig. 6) present, but this extraneous material did not seem to obscure either the final differential count or tinctorial properties of the stain.

The stratified squamous epithelium of the biopsy specimens was classified into four types of keratinization as described by Weinmann and Meyer (24) in their study of human gingiva. Their sections were also formalin fixed and stained with H & E and Mallory's stains in which the latter stains keratin a bright red color. The four types consisted of: (1) non-keratotic, (2) incomplete parakeratotic, (3) parakeratotic and (4) keratotic.

The filter imprint cytology of each of the 49 selected oral mucosal areas were divided into mean percentile groups of epithelial cells according to whether they were pre-cornified, cornified or keratinized. From these counts the type of surface epithelium was predicted. These predictions were then checked against the representative biopsy specimens, and it was found that the filter imprints, stained by Papanicolaou stain, can be used with relative accuracy in predicting surface characteristics of biopsy samples from the same area of the oral mucosa (Graph I and Table III).

A graphic representation of the mean percentages of the cells of the filter imprint cytology, stained by Papanicolaou's method, as compared to the classification of the four types of stratified epithelium of the Mallory stained biopsy sections as established by Weinmann and Meyer (24) is shown in Graph I. It can be seen that the mean percentages of pre-cornified cells decreases progressively from the non-

TABLE II

Mallory's connective tissue staining procedure

Solution or Stain	Biopsy Sections	Filter Imprints
Deparaffinized with changes from xylol through alcohols	Standard Procedures	None
Hydrate in 80%, 70% and 50% alcohols	None	2 minutes each
Rinse in distilled water	1 minute	2 minutes
3% potassium dichromate	2 hours minimum	2 hours minimum
Rinse in distilled water	2 changes	4 changes
0.1% acid fuchsin	15 seconds	20 seconds
Rinse in distilled water	2 changes	4 changes
1% phosphomolybdic acid	3 minutes	3 minutes
Rinse in distilled water	2 changes	4 changes
Blue Mallory II*	25 seconds	37 seconds
Rinse in distilled water	2 changes	4 changes
Dehydrate and clear by running through 50%, 75%, 95% and absolute alcohols	3–5 quick dips	3–5 quick dips
Mixture of absolute alcohol and xylol equal parts (1:1)	3–5 quick dips	None
1-propanol, mixture of 1-propanol and xylol equal parts (1:1)	None	30 seconds each
Xylol, two changes	5 minutes each	10 minutes each
D.P.X.†	Mountant	None
H.S.R.	None	Mountant

* Blue Mallory II solution: Orange G (10.0 gm.), Oxalic acid (10.0 gm.), Aniline blue (2.5 gm.) and distilled water (500 cc.).

† The British Drug Houses Ltd., B.D.H. Laboratory Chemicals Group, Poole, England.

keratotic to the keratotic class. Conversely, the mean percentages of the keratinized cells increase progressively from the non-keratotic to the keratotic class. The greatest mean percentage of cornified cells is found in the incomplete parakeratotic classification, (Graph I).

The means and ranges of the cytology of the Papanicolaou-stained filter adherent cells as compared to the four stages of keratinization of the normal oral mucosa in histologic sections is presented in Table III. It will be noted that the keratotic class includes cells that range from pre-cornified to fully keratinized.

Table IV shows the means and ranges of the three cell types for each of the various areas of the oral mucosa. The sample number of each area is also included in the table.

DISCUSSION

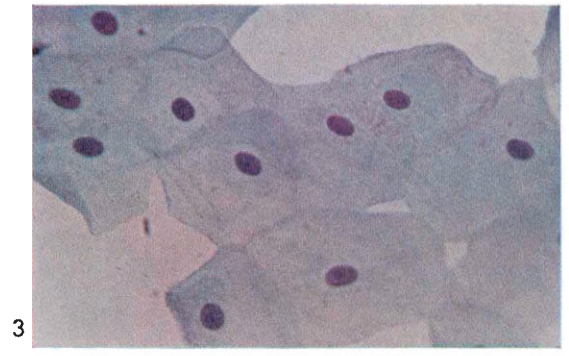
Cytologists generally use the terms "degrees of cornification" when describing degrees of cell maturation while histologists and pathologists tend toward the term "degrees of keratinization" to imply the same thing. It was felt that since the Papanicolaou stain produces three different colors in the cell cytoplasm, the term used in this study should be the same as Papanicolaou and his followers advocate, viz.,

a) *pre-cornified* cells which stain blue to green and have nuclei present,
b) *cornified* cells which stain pink to red and have nuclei present which sometimes tend to be pyknotic, and,
c) *keratinized* cells which stain yellow to orange and may have degenerating nuclei varying from pyknosis to karyolysis; in this group most of the cells are non-nucleated.

The term "parakeratosis" has also been debated.

"Parakeratosis: The retention of nuclei in the stratum corneum of the epidermis. Usually associated with some inflammation in the prickle-cell layer, resulting in a disturbance in the process of keratinization. Occurs normally in the stratified squamous epithelium of true mucous membrane."(25)

Parakeratosis is a term which although not



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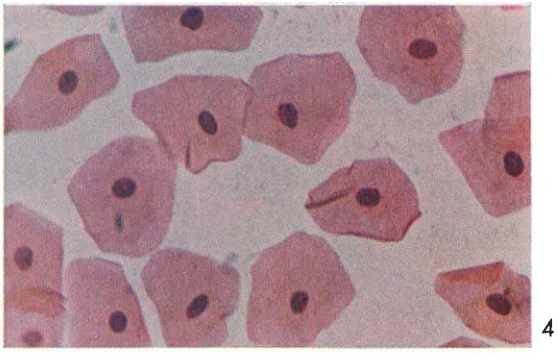

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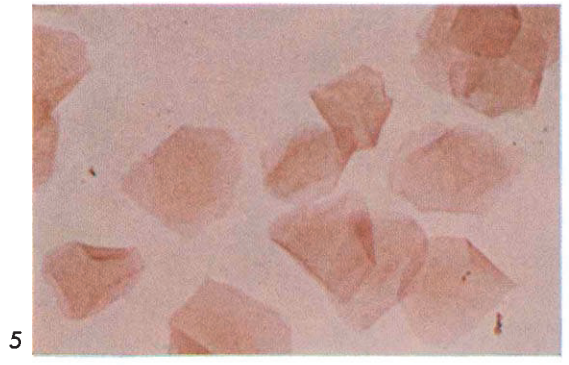

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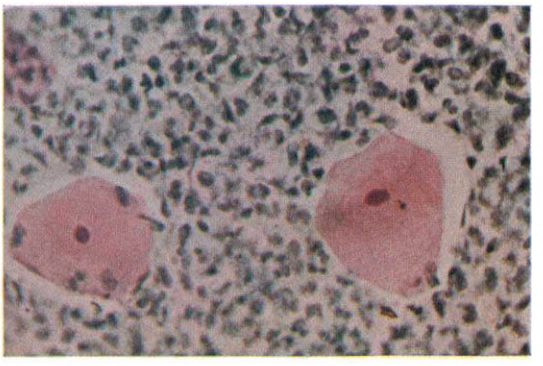

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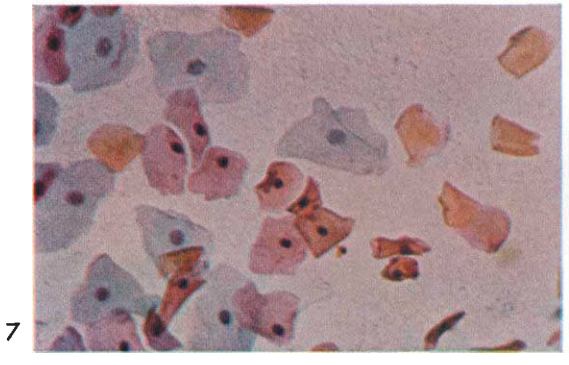

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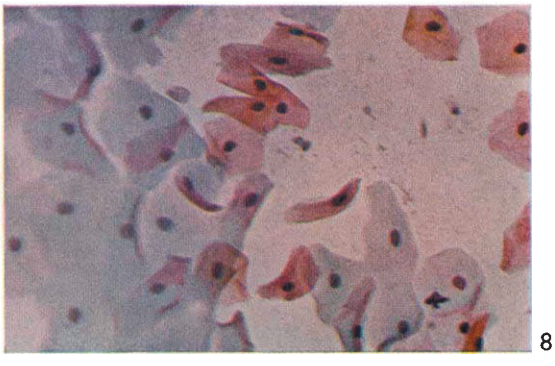

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FIGS. 3, 4, and 5 are photomicrographs showing selected surface epithelial cells on the filter after Papanicolaou staining. Note that the filter disc matrix does not interfere in the viewing of the stained cells. × 920.

3: Pre-cornified squamous epithelial cells in which the cytoplasm stains blue to bluish-green. Note that although there is some overlapping of the cells the number of cells present may be determined. Also, chromatin granules may be noted in the nuclei. This sample is from the buccal mucosa of a 16 year old, white, female.

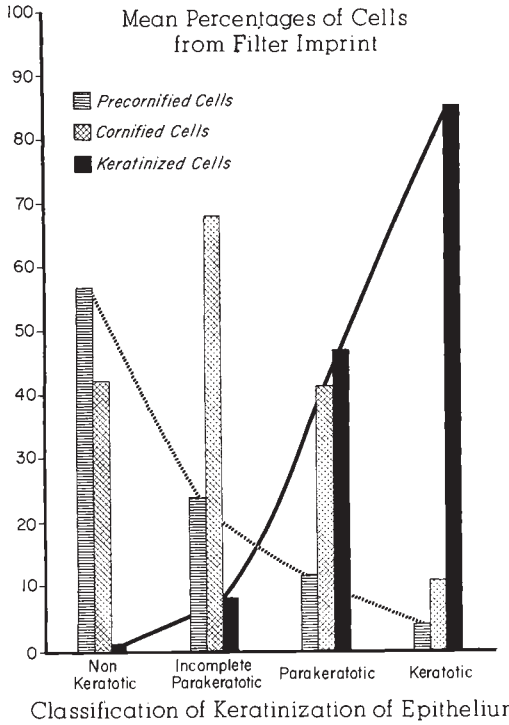
4: Cornified squamous epithelial cells in which the cytoplasm stains reddish. Note that several cells appear to be wrinkling slightly. This sample is from the attached gingival area of a 20 year old, white, female.

5: Fully keratinized squamous epithelial cells in which the cytoplasm stains orange. Note that the cells are anucleated and are more curled and wrinkled at the edges. This sample is from the hard palate, just lateral to the midline, of an 18 year old, white, male.

FIG. 6. Photomicrograph in which two cornified cells can be seen bathing in an inflammatory exudate. Note the reddish cytoplasm and the slightly pyknotic nuclei. This sample is from the free gingival area of a 13 year old, white male, at the right maxillary first pre-molar region. Papanicolaou stain. Oil × 2070.

FIG. 7. Photomicrograph shows varying proportions of all three types (pre-cornified, cornified and keratinized) of epithelial cells. Note that the blue granular background present is due to mucous residue that stains blue. Despite the overlapping of cells, the color differentiation and nuclear detail is quite distinct. This sample is from the attached gingival area of a 16 year old, white, male. Papanicolaou stain. × 550.

FIG. 8. Photomicrograph of a "zone of transition" of cell types that include the pre-cornified (left) to cornified cells (right). Note that the cornified cells are more wrinkled and curled than the pre-cornified cells and that a slight amount of oral debris is present. The sample is from the attached gingivo-alveolar mucosa junction of a 22 year old, white, male. Papanicolaou stain. × 550.



GRAPH I. A graph representing the mean percentages of the cells of the filter imprint cytology, stained by the slightly modified Papanicolaou's method, as compared to the classification of the four types of epithelium of the Mallory stained biopsy sections. The broken line shows the progressive decrease in precornified cells and the solid line shows the progressive increase in the keratinized cells. A further breakdown of the percentages of cells as established from the filter imprints is presented in Table III.

TABLE III

Comparison of degrees of keratinization of the oral mucosa by histological and cytological methods

Histologic	Cytologic		
Classification of epithelial surface*	Filter imprint cell type†	Percentages of cells	
		Mean	Range
Non-Keratotic	Precornified‡	56.8	44.0–69.0
	Cornified§	42.2	29.5–54.5
	Keratinized‖	1.0	0.0–2.0
Incomplete Parakeratotic	Precornified‡	23.9	9.5–45.5
	Cornified§	68.0	48.3–84.0
	Keratinized‖	8.1	2.5–16.2
Parakeratotic	Precornified‡	11.8	5.0–27.0
	Cornified§	41.3	22.5–58.0
	Keratinized‖	46.9	18.0–71.5
Keratotic	Precornified‡	4.0	0.9–9.7
	Cornified§	10.9	3.0–21.7
	Keratinized‖	85.1	68.6–96.1

* Mallory stained.
† Papanicolaou stained.
‡ Blue-green cytoplasm.
§ Pink-red cytoplasm.
‖ Yellow-orange cytoplasm.

generally used by cytologists is used in both the dental and other medical literature. In this connection, parakeratosis is a normal manifestation found in certain areas of the oral mucosa.

The first use of the term "incomplete parakeratosis" in the literature was by Weinmann and Meyer (24) in their study of the types of keratinization in the human gingiva. Their findings are supported in the present study because the same type of staining reaction occurred not only in some cases in the gingiva but also in the cheek and lower lip epithelium as well.

It is to be emphasized that any differential count will include varying proportions of all three types (pre-cornified, cornified and keratinized) of epithelial cells (Fig. 7). Whether a keratinized cell contained a nucleus or not played a minor role in the differential counts of this study because in the preliminary counts only 4.3% (mean percentage) were nucleated. Thus the cytoplasmic color was the determining differential factor of the type of superficial epithelial cell. The predominating cell in each differential count correlated very well as to Weinmann and Meyer's classification using Mallory's stain (24).

It was interesting to find a diminished number of cornified cells with pyknotic nuclei as compared to normal findings in the vaginal smears (23). Perhaps the nuclei of epithelial cells of the oral mucosa maturate according to a different pattern than in the vaginal and cervical surface cells.

Two consecutive filter imprints were routinely taken throughout the study, although not included in this study, additional consecutive imprints were performed on the buccal mucosa in several cases. As many as 12 consecutive

TABLE IV

Differential cell counts of specified areas of normal oral mucosa

Area Examined	Sample Number	Cell Type of Papanicolaou Stained Filter Imprint	Percentages of cells	
			Mean	Range
Buccal	16	Precornified*	51.1	27.5–69.0
		Cornified†	46.2	29.5–67.5
		Keratinized‡	2.7	0.5–6.5
Lower Lip	8	Precornified*	38.8	9.5–65.2
		Cornified†	58.7	34.5–83.7
		Keratinized‡	2.5	0.0–8.0
Lower Lingual Alveolar	5	Precornified*	16.3	10.2–27.0
		Cornified†	68.8	22.0–80.2
		Keratinized‡	14.9	9.3–18.0
Free Gingiva	4	Precornified*	21.4	7.5–44.5
		Cornified†	55.6	30.5–74.5
		Keratinized‡	23.0	5.0–62.0
Attached Gingiva	7	Precornified*	8.8	5.0–14.7
		Cornified†	39.4	15.5–58.0
		Keratinized‡	51.8	35.3–79.3
Alveolar Edentulous Ridge	4	Precornified*	11.7	3.7–19.5
		Cornified†	33.9	12.3–60.7
		Keratinized‡	54.4	25.0–84.0
Hard Palate	5	Precornified*	2.1	0.9–6.0
		Cornified†	8.4	3.0–22.5
		Keratinized‡	89.5	71.5–96.1

* Blue-green cytoplasm.
† Pink-red cytoplasm.
‡ Yellow-orange cytoplasm.

imprints were made with diminishing number of cells for each sample.

The standard Papanicolaou staining procedure had to be slightly modified for better differential staining quality of the cells. This modification included an increase in exposure time and acid concentration during the steps indicated in Table I. Propanol was found to be superior to absolute ethyl alcohol in preventing cloudiness of the filter during dehydrating and clearing processes.

The Mallory staining procedure used for the histologic sections was also slightly modified for staining the filter imprints. This modification also included an increase in exposure time during the steps indicated in Table II, for the same reasons as in the Papanicolaou procedure.

The use of a notched filter disc in sampling a mucosal surface produces an oriented filter imprint. For example, the microscopic examination of such an imprint from the gingival mucosa will reveal three transitional areas: (1) cornified epithelial cells plus polymorphonuclear leukocytes in the free gingival area, (Fig. 6), (2) cornified and keratinized cells in the attached gingiva, and (3) pre-cornified cells in the alveolar mucosa. One such area of transition from pre-cornified to cornified cell types is shown in Figure 8.

Most investigators either fail to mention the range in their differential counts or else they report counts that are small, such as "a random cell count of 100 cells" (7), or, the reports are of "counting the first hundred cells encountered which occurred singly or in small clumps" (8).

In the beginning of this study it was determined that for a differential count to be significant, a minimum of 400 cells and a maximum of 1,000 cells be counted for each representative sample.

The literature reveals that although significant and extensive studies have been described for both the cytology and histology of the oral mucosa, there apparently has not been a comparative study devoted to both cytologic and histologic methods of evaluation of the epithelial lining on selected areas of the *normal* oral mucosa. In this study the Millipore filter was used as a cell adherent vehicle for a cytologic determination of degrees of keratinization of the normal oral mucosa. This filter imprint technic was found to be a reliable method for obtaining superficial epithelial cells, and the findings as to the type of keratinized surface correlated very well with those of histologic sections of mucosa of the same area.

SUMMARY AND CONCLUSIONS

1. Superficial cells of the normal oral mucosa were successfuly removed by the use of Millipore filters following direct contact with the surface of the mucous membrane.

2. Tinctorial properties and cytologic detail was sufficient to distinguish between the stages of maturation of superficial epithelial cells attached to the filter discs.

3. The cells on the filter were classified into pre-cornified, cornified and keratinized types according to Papanicolaou's criterion and the mean percentages were determined.

4. Forty-nine filter samples on forty-one normal individuals were taken of selected areas of the oral mucosa. The same mucosal areas were immediately removed for biopsy study, fixed, processed and the tissue sections were stained with H & E and Mallory's stains.

5. Comparison between the filter imprints and biopsy specimens from the same area revealed a reliable correlation between the two methods of observation.

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