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FINE STRUCTURE OF SUBCULTIVATED STRATIFIED SQUAMOUS EPITHELIUM GROWN ON COLLAGEN RAFTS

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SUMMARY

Subcultivated rat lingual epithelial cells when grown on collagen gels at a liquid–gas interface achieve a highly ordered state that closely resembles the parent tissue. Three distinct cell layers are present; basal, spinous, and keratinized. Basal cells are cuboidal in shape and form a complex interface with the underlying collagen fibrils. Spinous cells form a layer 5–10 cells thick and, with the exception of keratohyalin granules, possess an organellar complement identical with native cells, including membrane-coating granules. The keratinized cell layer increases in thickness as a function of time spent in culture. Forty or more plies of terminally differentiated cells are observed following a 30-day culture period. Terminally differentiated cells while retaining pycnotic nuclei and some other organellar debris are principally envelope-enclosed squames filled with tonofilaments. Keratinization is a continuing process which occurs simultaneously across the full expanse of the culture surface. The high degree of tissue organization observed appears to be the result of feeding the cultures from the undersurface.

Collagenous substrata facilitate the growth of fastidious cells in culture [1, 2]. They have also proved useful in culture systems where maintenance of a highly differentiated cell type was an objective [3, 4]. We have employed collagenous substrata in the culture of subcultivated rat lingual epithelium, the nature of which, in conventional culture, is described in the preceding paper [5]. When such cultures are supported by collagen rafts at a liquid–gas interface, the cells organize and function in a manner closely resembling that of the parent tissue. The results obtained with this culture system are the subject of this report.

MATERIALS AND METHODS

Cell cultures

Rat lingual epithelial cells in passage 5, 6 or 7 were used in this study. The cells were all derived from the same primary culture. The methods of culture and subculture are described in the preceding paper [5]. Briefly, the cells were grown in plastic culture flasks and were supplied with complete culture medium, which consisted of Minimum Essential Medium with Earle's salt mixture (MEM), 20% (vol/vol) fetal calf serum (FCS), 50 μg/ml Gentamycin sulphate, and 0.5% (vol/vol) dimethyl sufoxide (DMSO). The incubation temperature was 32°C and the gaseous environment was 95% air 5% CO_2.

Preparation of the collagen gel

Collagen was extracted from guinea pig skin [6] following the method of Kang et al. [7]. Briefly, the method involved extracting shaved, minced guinea pig skin (200–500 g body weight) in 20 vol of 1 M NaCl, 0.005 M benzamidine–HCl, 0.05 M Tris-HCl, pH 7.5, per g wet weight of skin for 24 h at 4°C. All subsequent steps were carried out at 4°C. The salt-extracted tissue was then extracted overnight in 20 vol of 0.5 M acetic acid/g of original tissue wet weight. The extract was filtered through coarse nylon mesh and clarified by centrifugation at 27000 g for 30 min. Sodium chloride was added to the clarified supernatant to a final concentration of 20%. The resulting precipitate was collected by centrifuging at 27000 g for 45 min. The acetic acid solubilization and salt precipitation was repeated an additional time. The precipitate was then redissolved in 0.5 M acetic acid, clarified by centrifugation at 105000 g for 1 h before being dialysed

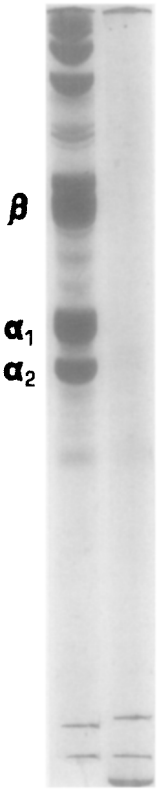


Fig. 1. SDS polyacrylamide gels of the collagen used to form support substrata. The gel on the left contains collagen incubated in buffer, the gel on the right was loaded with an equal amount of collagen following collagenase digestion. In addition to the alpha and beta banding characteristic of Type I collagen, several minor bands are present. All the protein present on the left gel was degraded by collagenase indicating the bands represented collagen or collagen fragments. Protein markers when included in the collagenase digestions were not degraded.

against several changes of 0.2 M sodium phosphate, pH 7.2. The resulting precipitate was dissolved in 0.5 M acetic acid, dialysed against several changes of 0.1 M acetic acid, clarified (105000 g), and lyophilized. The collagen was stored at 4°C in the presence of a drying agent.

Purity of the preparation was determined by polyacrylamide gel electrophoresis, and the collagenous nature of the apparent contaminants (minor bands) was demonstrated by collagenase digestion. Lyophilized collagen was dissolved in 0.04 M Tris-HCl, 22 mM $CaCl_2$, pH 7.5. To an aliquot of the solubilized sample, collagenase (Form III, Advanced Biofactures Corp., Lynbrook, N.Y.) was added to give a concentration of 10 moles % with respect to collagen. *N*-Ethymaleimide (NEM) was added to all samples (16 mM final

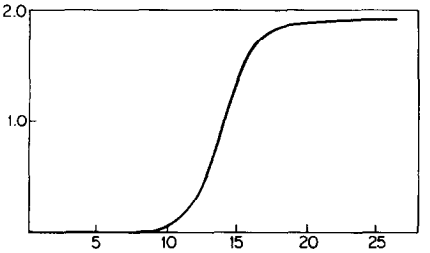


Fig. 2. Abscissa: time at 37°C (min); *ordinate:* A_{475}. An optical absorbance curve (475 nm) indicating the rate of fibrillogenesis of guinea pig skin collagen. Curve is for a solution containing 3 mg collagen/ml.

concentration) and the resultant mixture incubated at 37°C for 3 h [8]. The control contained buffer instead of collagenase. The enzyme catalase was added (0.5 mg/ml) to some samples in order to check for non-specific proteolytic digestion by the collagenase and to provide a molecular weight indicator. The reaction was stopped by adding sodium dodecyl sulfate (SDS) to a final concentration of 2% and a 2-fold excess of dithiothreitol (DTT) with respect to NEM and heating at 70°C for 30 min. The preparations were dialysed overnight against 0.05 M Tris-H_2SO_4, pH 6.1, and 0.5% SDS. Polyacrylamide tube gels (5% T, 2.7% C) were intentionally overloaded with 60 μg of collagen, in order to detect trace contaminants. The gels were run in a Tris-glycine buffer system (0.05 M, 0.1% SDS) at 2 mAmp/tube. The gels were stained with Coomassie Brilliant Blue R-250 [9].

The stained gels (fig. 1) demonstrated several minor bands in addition to the major alpha and beta (and gamma) forms typical of Type I skin collagen. All bands demonstrated the metachromatic staining characteristic of collagen [10] and all were absent from the gel containing the collagenase-digested protein. The fact that degradation of a non-collagenous protein (catalase) was not observed leads us to conclude: (1) the enzyme does not contain appreciable amounts of other proteases; (2) the minor bands probably represent collagen partially degraded by proteases during the isolation procedure [11]; (3) significant amounts of non-collagenous protein are not present in the collagen preparation.

To form collagen gels, collagen (4 mg/ml) was dissolved in 0.1 M acetic acid. The collagen was then dialysed against two changes of 50 vol of 0.2 M NaCl, 0.5 M Tris-HCl, pH 7.5, for a total time of 24 h at 4°C. The original volume was re-established by adding dialysis buffer and Gentamycin sulphate to yield a final concentration of 100 μg/ml. The 4°C collagen solution (1.5 ml) was added to 60 mm plastic culture dishes (Falcon 3002; Falcon Plastics, Oxnard, Calif.). The dishes were placed on a level surface in a humidified incubator (37°C) and left undisturbed for 12–18 h. The onset and rate of fibrillogenesis was followed by monitoring the change in absorbance (47.5 nm) that occurs when the solution is warmed to 37°C [12, 13] (fig. 2). Collagen solutions that did not exhibit maximum fibril growth (ΔA^{max} 475, 3 mg/ml) by 30 min were not used. The concentration of the Gentamycin used

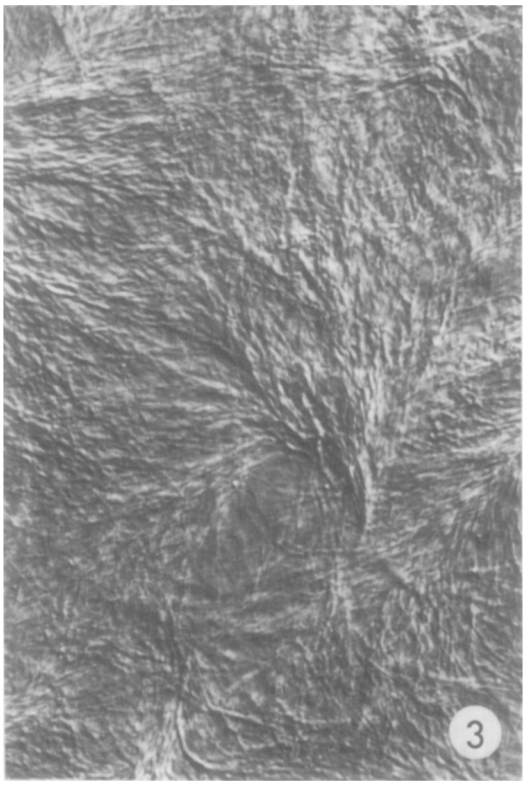


Fig. 3. Differential interference contrast micrograph of formed 0.6% collagen gel. Gels characteristically demonstrated bundles of collagen with a central condensed region from which fibrils spread out into the surrounding area forming a mat. ×770.

did not alter fibril formation. The gels were subsequently conditioned by several changes of complete culture medium. Cells used in this study behaved identically on gels ranging from 0.4–0.8% collagen. The concentration of 0.6% was chosen because the viscosity of the collagen solution permits relatively easy solution transfer, and the resultant gels can be readily manipulated.

Morphology of the collagen gels

The structure of the gel consisted of numerous, randomly ordered bundles of collagen fibrils. Fibrils spread out from one or both ends of a central, condensed region of the bundle (fig. 3). When the gels were studied by scanning electron microscopy (SEM) (glutaraldehyde osmium-fixed, CO_2 critical point-dried, gold–palladium sputter-coated), the fibrillar nature of the gel was further emphasized (fig. 4). Collagen fibrils, most of which ranged between 0.1 and 0.2 μm in diameter, overlapped one another, forming a meshwork of considerable depth.

Preparation of basally fed cultures

Rat lingual epithelial subcultures in the 5th, 6th, or 7th passage were removed from 30 cm^2 flasks by incubating the cells at 4°C for 60 min in 0.25% trypsin and 3.2 mM EDTA as described in the accompanying paper [5]. The contents of one flask ($8–10\times10^6$ cells) were divided and added to two conditioned collagen gels in 60 mm (28 cm^2) plastic tissue culture dishes. The subcultures on collagen were grown in complete medium without DMSO for 5 days. On day 5, the collagen gels were cut into triangular pieces having sides approx. 1–1.5 cm. Only that portion of the gel which occupied the central two-thirds of the culture dish was used. The gel thickness ranged from 25 to 100 μm. The gels were floated onto stainless steel organ culture grids (Falcon 3014) which were then placed in plastic organ culture dishes (Falcon 3037). The final relationship of the cells, collagen, support, and medium is illustrated schematically in fig. 5. The cultures were maintained in a humidified atmosphere at 32°C with the medium being replaced three times each week. During medium changes, care was taken not to disturb the culture surface.

Electron microscopy

Cultures were fixed on the organ culture grids in a mixture of 1 part 2.5% glutaraldehyde and 1 part 2% aqueous OsO_4. Following fixation, dehydration, and infiltration in Epon-Araldite, the collagen gels were cut into smaller pieces and placed in flat embedding molds. Cultures were fixed 5 days following plating (day 0 of lifting) and 10, 17, and 31 days after lifting. Replicated (control) cultures were grown on collagen gels which were not lifted. The findings presented here are based on three independent experiments conducted over a period of 6 months.

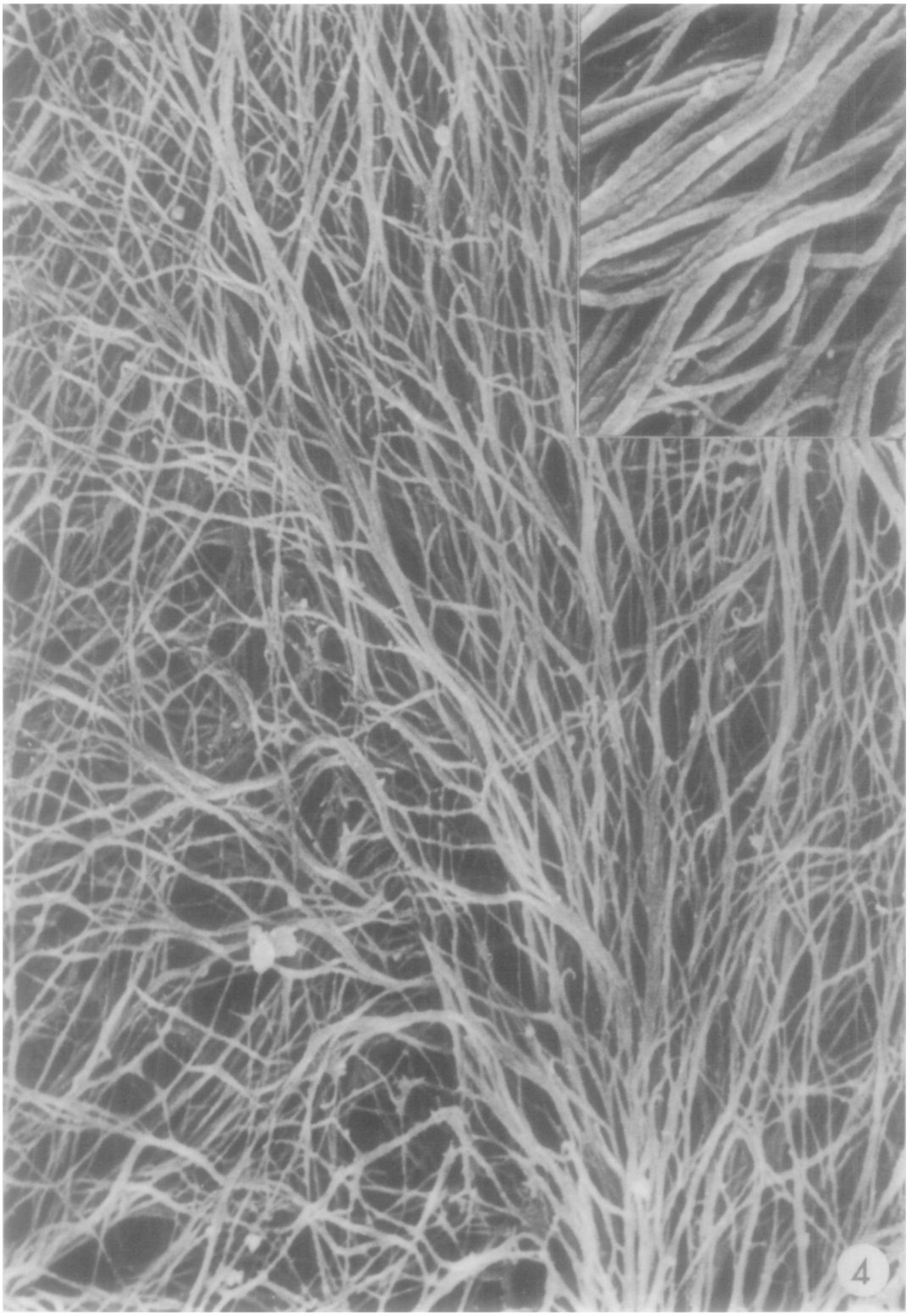
Solubilization of surface cells and histochemical procedures

Cultures were immersed in trypsin–EDTA at 34°C for 10 min. The cells were dispersed by agitation and collected by centrifugation. They were resuspended and then extracted overnight at 50°C in 2% SDS in 0.1 M Tris-HCl, pH 8.6, with or without 8 mM DTT. The cells were collected by centrifugation and examined using differential interference contrast optics. Cultures for histochemical study were fixed in 10% neutral formalin containing an organomercurial compound, Mersalyl (K&K Laboratories, Plainview, N.Y.), dehydrated, and embedded in paraffin. Sections (7 μm) were then processed and sequentially stained, resulting in the simultaneous demonstration of native thiol and disulphide groups [14, 15].

RESULTS

Light microscopy

Examination of 1 μm thick sections by light microscopy revealed that the cells grown on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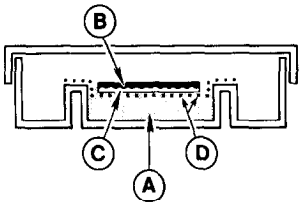


Fig. 5. A schematic illustration of a raised epithelial cell culture. *D*, Stainless steel support grid; *C*, collagen gel; *B*, epithelium. The medium (*A*) level is adjusted so it will just be drawn onto the surface of the culture.

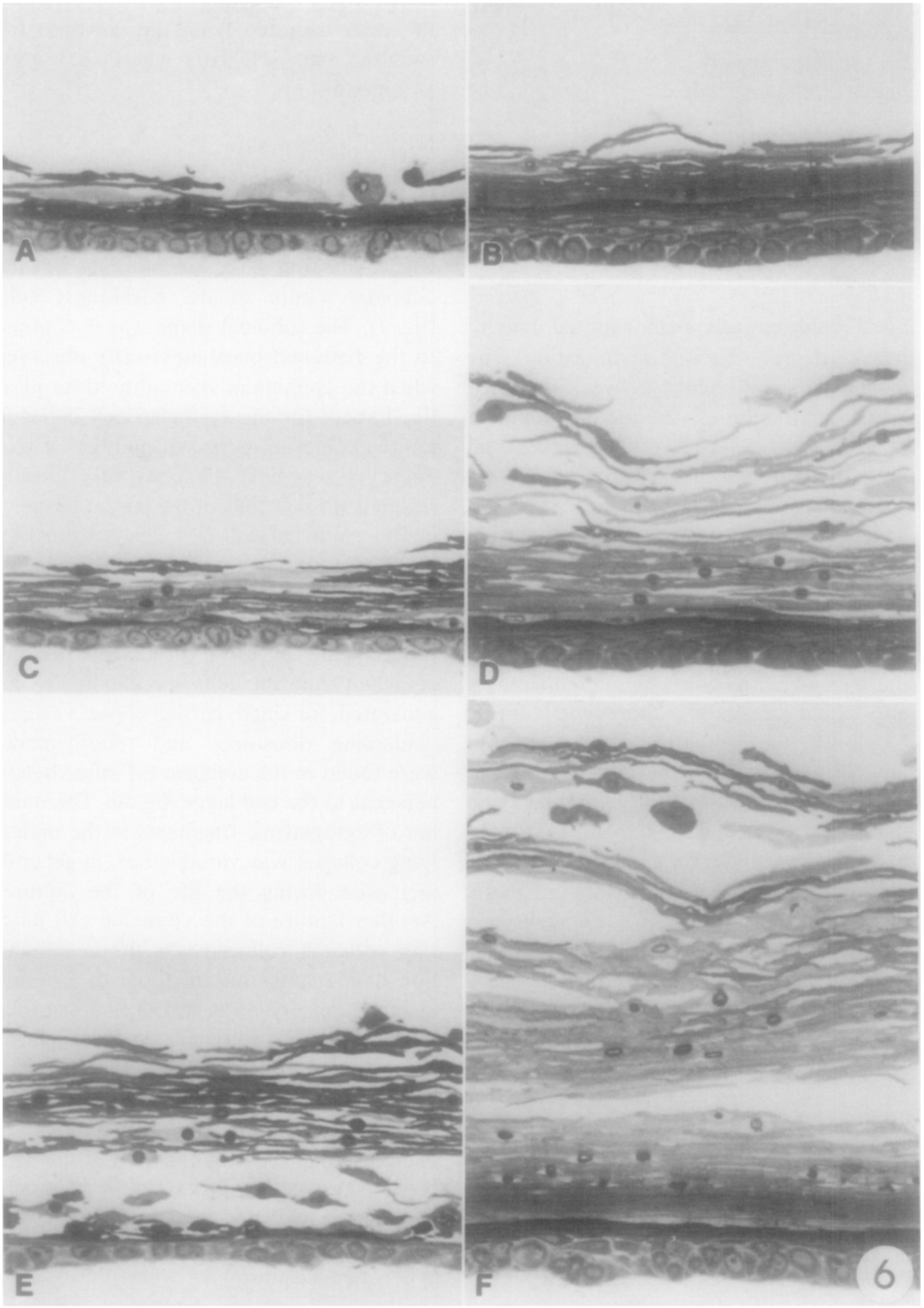
lifted collagen gels demonstrated an enhanced degree of tissue organization. The most obvious difference between the lifted and unlifted cultures was the development of an extensive (5–10 layers) suprabasal cell layer (which will be referred to as the spinous cell layer.) Another difference, which was not apparent from viewing a section, was that of a simultaneous development of a keratinized layer across the entire surface of the lifted cultures. The pankeratinization of lifted cultures versus the spotty keratinization of unlifted cultures also could be readily appreciated during fixation by the degree and pattern of osmium blackening that occurred. The keratinized layer was more tightly organized and thicker in lifted cultures than that found in unlifted ones (fig. 6*a–f*). Thirty-one day-old cultures did demonstrate foci of tissue disorganization, including intraepithelial keratinization, and for this reason the study was not extended to longer time periods. Limited (0.5 mm) circumferential growth of cells around the collagen gel occurred and the cells migrated from the gel onto the stainless steel grid bars where they grew as a monolayer.

Fig. 4. SEM of a collagen gel. Micrograph demonstrates a region where the fibrils are spreading out into the surrounding area forming a meshwork with other collagen fibrils. The particulate material in the gel is precipitated serum protein. Epithelial cells similar to those illustrated in fig. 7 would occupy approx. 25% of the area illustrated in this micrograph. *Inset.* Higher power SEM of a collagen gel depicting a recurring (~ 150 nm) striated or beaded pattern. Both ~70 nm and ~150 nm banding patterns are observed in the formed fibrils with the SEM. ×8 000; *inset* ×17 000.

Electron microscopy

Ultrastructural study of the cultures emphasized the structural similarities of the cultured cells with those of the parent tissue. The first of these similarities was the cuboidal nature of the basal-most cells (fig. 7). The cuboidal shape was in contrast to the flattened basal-most cells observed when the epithelium was cultured on plastic. Except for the epithelial cell–collagen interface and an increased number of secondary lysosomes, the cells very closely resembled basal cells of the parent tissue.

The epithelial cell–collagen gel junction was highly complex with the epithelial cells sending out numerous, irregularly shaped processes into the underlying gel. Apparently some of these processes became detached from the cells and eventually degenerated, for small, partially lysed vesicles containing ribosomes and tonofilaments were found in the collagen gel immediately adjacent to the cell layer (fig. 8). The number of cytoplasmic fragments in the underlying collagen was variable but, in general, increased during the life of the culture. Another feature of the epithelial cell junction with the collagen was the accumulation of a filamentous material of variable electron opacity on both the undersurface of the epithelial cells and on the surfaces of subjacent collagen fibrils (fig. 9). Collagen fibrils greater than 1 μm distant from the epithelial cell undersurface were rarely coated. While the accumulation of this material was variable in extent, it was cell-associated and not observed in serum-conditioned gels before cells were added. When hemidesmosomes were present, the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material appeared to be preferentially deposited opposite them.

In contrast to unlifted cultures, lifted cultures demonstrated an increase in the number of spinous cells and a more robust development of the tonofilament–desmosome apparatus. It was often difficult to distinguish between micrographs of native and cultured spinous cells if the presence of secondary lysosomes in the cultured cells were ignored. Spinous cells in lifted epithelial cultures also demonstrated small, spherical or elliptical vesicles adjacent to the plasma membrane (fig. 11). The content of these vesicles was often distorted; however, the lamelliform character of the vesicular content could occasionally be observed (fig. 11). Such vesicles occurred in all spinous cell layers but were increasingly evident in the upper cell strata. In fortuitous sections, material resembling the lamelliform content of the vesicles could be observed between cells at the zone of transition between non-keratinized and keratinized cells (fig. 10). This osmiophilic material was also present between the first several layers of keratinized cells. Profiles suggesting discharge of vesicular contents were rarely observed. Often the 3–4 cell layers immediately subjacent to the keratinized cell layer showed cells with a markedly condensed cytoplasm which made ultrastructural study of this region difficult. This zone of condensation could also be recognized in 1 μm thick sections viewed with the light microscope (fig. 6*b*, *d*, *f*).

The keratinized cell layer was composed of up to 40 plies of tightly packed cells and the layer increased in thickness with the age of the culture. Desmosomes decreased in number in the upper cell layers but did not undergo the series of transformations observed during terminal differentiation in vivo [16, 17]. Degenerating nuclei and mitochondria, secondary lysosomes, and other inclusions, the lineages of which could not be established, were observed in all the keratinized cell layers (fig. 12). All keratinized cells demonstrated a thickening of the inner aspect of the cell membrane (envelope) and a cytoplasm filled with tonofilaments.

Histochemistry and solubility of keratinized cells

When paraffin sections were reacted for native thiols (blue) and disulfide bonds (red) using a technique in which the underlying reactions approach unit stoichiometry [15], the epithelium stained blue but changed abruptly to red at the beginning of the keratinized cell layer. Admixing of the colored reaction products at the junction of non-keratinized cells with keratinized cells was limited. The contents of the keratinized cells did not appear to dissolve when extracted with SDS. However, when a reducing agent was added they dissolved, leaving only the insoluble envelope, which could be easily visualized by differential interference contrast microscopy.

DISCUSSION

Compared with cultures grown on a plastic culture surface, an enhanced degree of tissue organization is observed if subcultivated rat lingual epithelium is grown on a

Fig. 6. Comparison photomicrographs of subcultivated epithelium grown on unlifted and lifted collagen gels. *A*, *B* compare 10-day-old cultures, *C*, *D*, 17-day-old cultures, *E*, *F*, 31-day-old cultures. The increased development of the suprabasal cell layer in lifted culture is evident. The number of terminally differentiated cells, most of which have retained a nucleus, increased during the duration of the culture. A zone of darkly staining cells is evident at the transition from non-keratinized to keratinized cells in the lifted cultures. ×330.

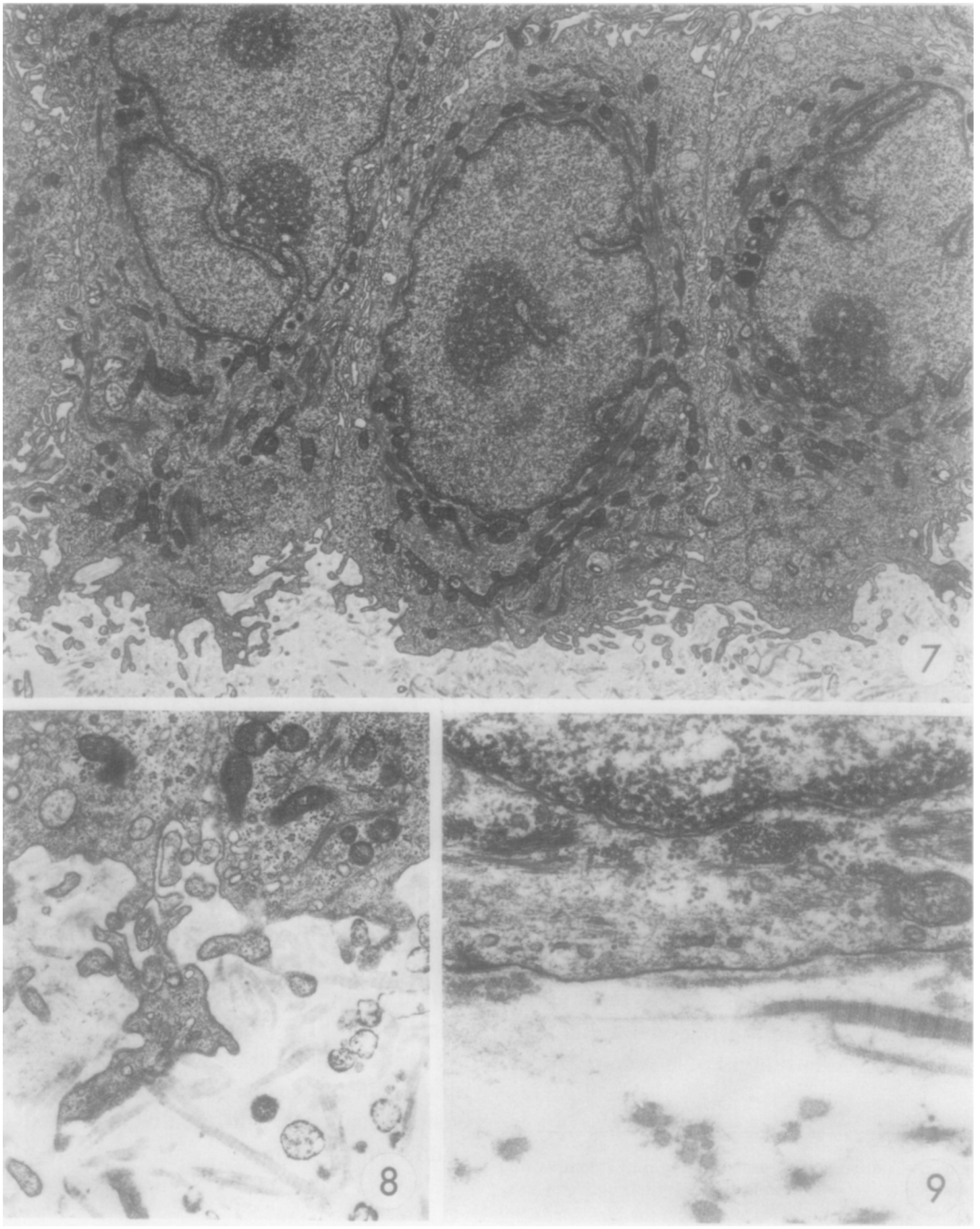

Fig. 7. Electron micrograph of basal-most cells in a lifted 10-day-old culture. The cells have a cuboidal or columnar shape similar to that of native basal cells. The cells send out slender cytoplasmic processes into the subjacent collagen fibrils. ×6 550.

Fig. 8. Electron micrograph of an epithelial cell junction with the collagen. Cytoplasmic processes as well as degenerating cell fragments are present among the collagen fibrils. ×16 700.

Fig. 9. An accumulation of filamentous material on the surfaces of collagen fibrils as well as on the undersurface of an epithelial cell is illustrated. ×35 000.

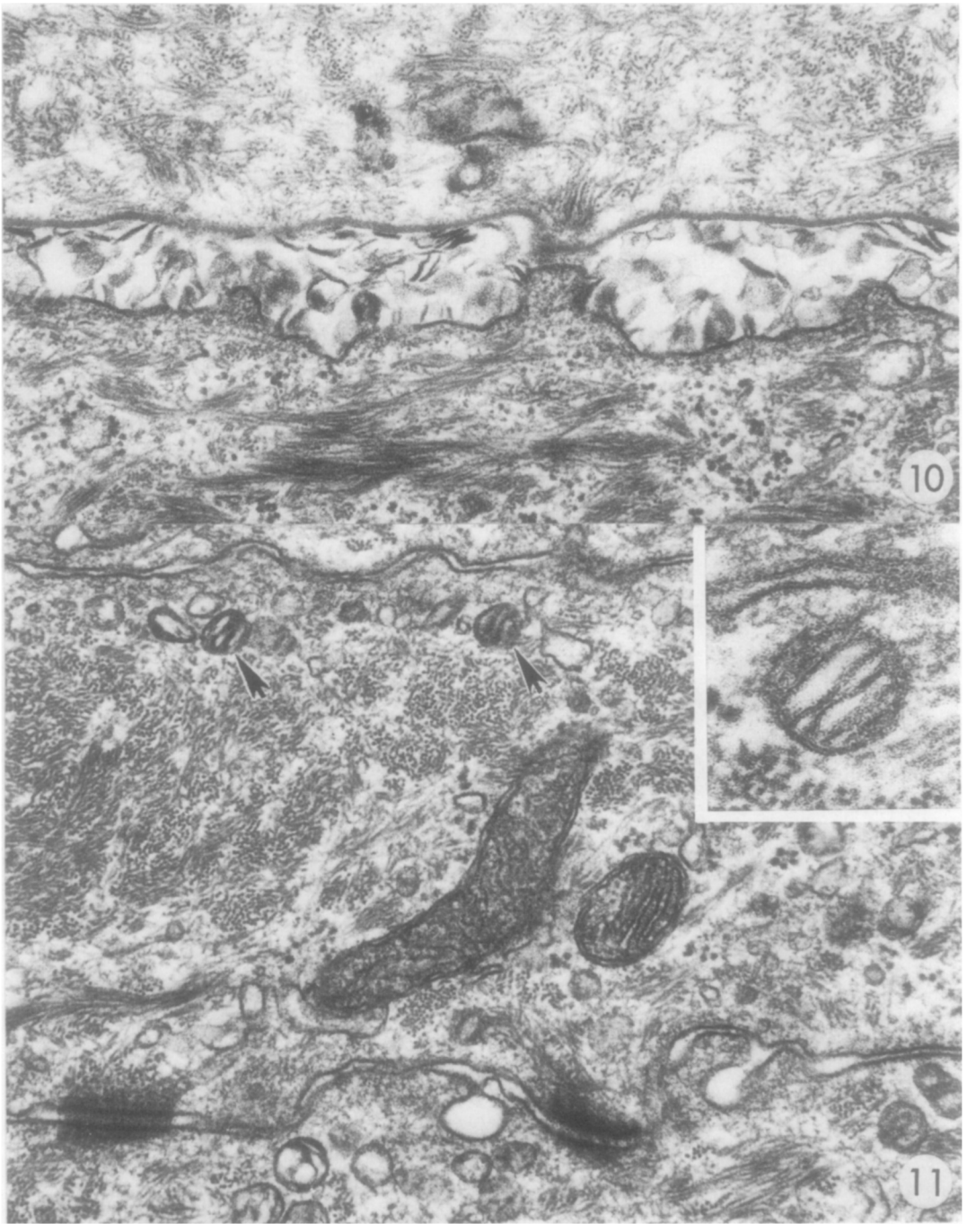

Fig. 10. Lamelliform particles are present in the extracellular space between a non-keratinized and keratinized cell. This material resembles the product contained in spinous cell vesicles (see fig. 11). The keratinized cell demonstrates envelope formation. ×56 000.

Fig. 11. Vesicles (*arrows*) in the cytoplasm of a cultured spinous cell. *Inset.* Higher power micrograph of spinous cell vesicle illustrating the lamelliform content. ×62 000; *inset*, ×137 500.

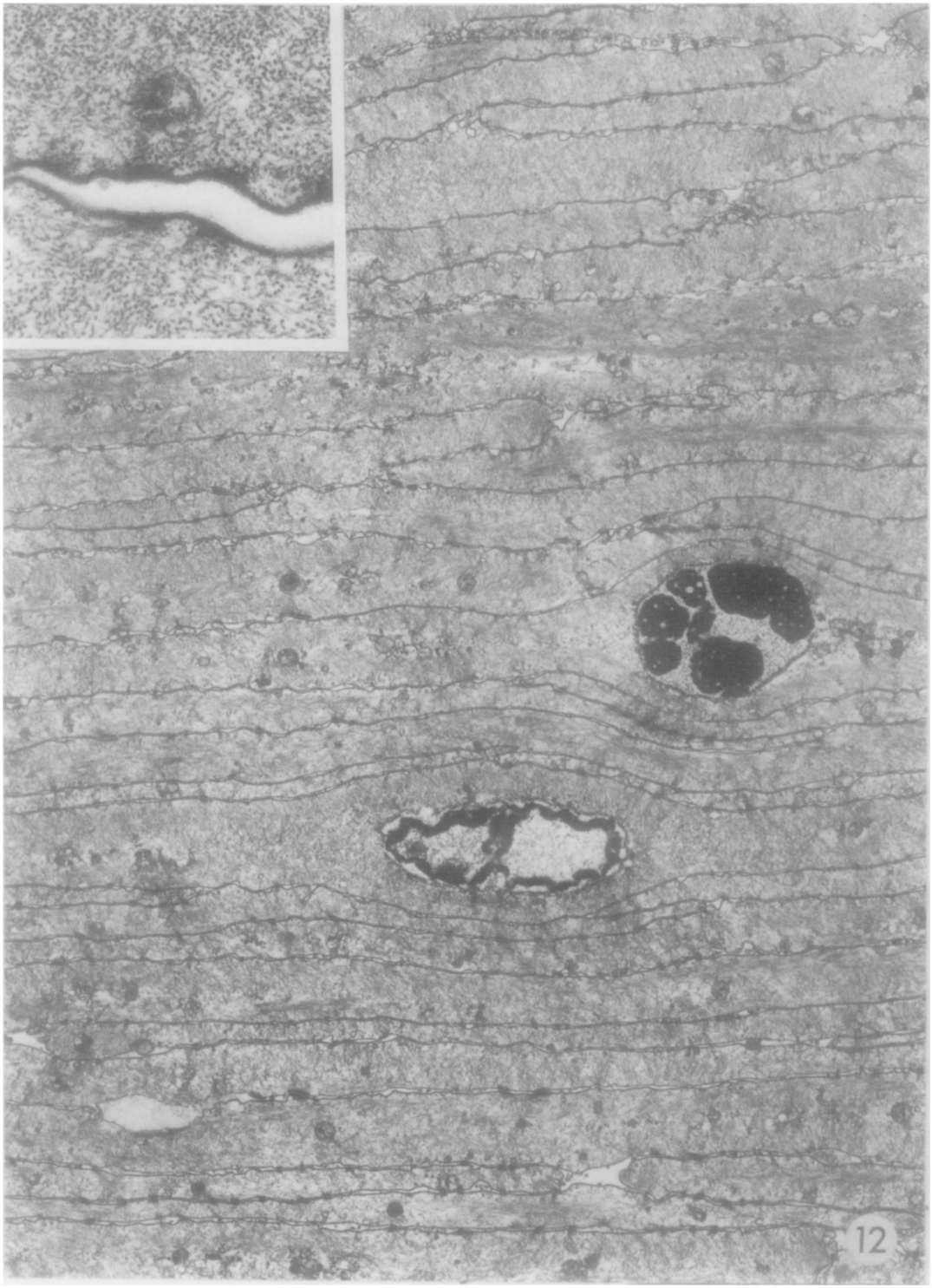

Fig. 12. Survey electron micrograph of terminally differentiated (keratinized) cells. Note the pronounced layering and tight packing of the cells. Two pycnotic nuclei and assorted degenerating cell organelles are present in the cytoplasm of the cells. *Inset.* Higher power micrograph of two keratinized cells illustrating membrane envelopes and tonofilaments. *Inset,* ×6900; ×61000.

collagen raft maintained at a liquid–gas interface. The form of the basal cells, the presence of a well developed spinous cell layer, and the development of an extensive, ordered keratinized cell layer, all serve to emphasize the similarity of the cultures that are derived from dissociated cells with the parent tissue, stratified squamous epithelium. The principal advantage of this technique appears to be that the cultured cells are fed from underneath. The basal feeding advantage is best illustrated by comparing lifted with unlifted cultures of the same age. The advantage of basal feeding of a tissue that develops a sizeable surface coat of dead cells in vitro should be obvious. The parent tissue also derives its nutrients from blood vessels in the connective tissue, i.e., it is fed from below, with the metabolically active cell population being the first to receive nutrients. Further support for the concept of basal feeding being responsible for the heightened tissue development observed in this study comes from the fact that DMSO was not required in the culture medium to sustain growth. The lack of a DMSO requirement in lifted cultures was responsible for our conclusion that DMSO may act as a carrier (through the keratinized cell layer) of molecules essential for the survival of rat lingual epithelium in primary culture and subculture [5]. Addition of DMSO to the culture medium of unlifted cultures does not, however, increase the degree of tissue organization above that observed in cultures fed in the absence of DMSO.

Basal-most epithelial cells grown on a plastic culture surface are flattened or squamoid in appearance [18, 19, 5]. The return of the basal-most cultured epithelial cells to a shape resembling the basal epithelial cells of the parent tissue appears to be due to the properties of the collagen gel. The cuboidal or columnar form of the basal-most cells was observed in cultures initiated on either unlifted or pre-lifted collagen gels, thus obviating changes in cell shape that might have been engendered by cutting out and repositioning the gel during the lifting procedure (unpublished observation). The fibrillar nature of the substratum may present a physical environment more conducive to the close ordering of cells as opposed to smooth, high affinity plastic culture surfaces which promote cell spreading [20]. Fibroblasts are reported to remain more compact when grown on collagen gels as compared with the typical spreading behavior exhibited on tissue culture plastic [12]. Collagen gels bearing attached cells do not contract when placed on stainless steel grids as they do when allowed to float free in the medium ([3, 4] and this study, unpublished). Therefore it appears that the morphology of the cultures as presented in this study has not been significantly changed by alterations in gel shape and size. We have no indication that collagen has a direct influence on rat lingual epithelium in vitro beyond that of influencing the shape of the basal-most cells. Since comparable substrata lacking collagen do not exist, it is extremely difficult to design experiments to check the effects of the collagenous raft. Preliminary observations of epithelium grown on Type III collagen or bovine Descemet's membrane yield essentially the same results as those obtained with Type I guinea pig skin collagen.

Many epithelial sheets send out processes (blebs) into the surrounding environment when removed from a basal lamina [21]. This same type of behavior has been observed in vivo when epithelial cells are positioned opposite defects in a basal lamina [22]. Results from the present study affirm this pseudopodal behavior of rat

lingual epithelial cells when cultured upon a reticular substratum. The detachment and subsequent degeneration of cell processes within the collagen gel does not appear to be deleterious to the cultured cells. Whether such blebbing behavior of the epithelial cells can be altered by filling the space between collagen fibrils with connective tissue molecules of large hydrodynamic size is the subject of a current investigation. The material deposited upon the undersurfaces of the basal-most cells and around collagen fibrils resembles the basal laminae deposited in culture by vascular endothelium [23], mammary epithelium [4], salivary gland epithelium [24], and recombined epidermis [25]. The deposition of this material by rat lingual epithelial cells in culture appears to be more erratic than that reported for the aforementioned systems. The conditions responsible for this erratic deposition are unknown.

The vesicles with the lamelliform content observed in cultured spinous cells are identical to membrane-coating granules (MCGs), observed in native stratified squamous epithelium [26, 27, 28]. Similarly the material between cells at the zone of transition between non-keratinized and keratinized cells is interpreted to be the released product of the vesicles. The location of the extracellular material parallels the location of the discharged contents of membrane-coating granules in vivo. To our knowledge, MGCs have not been described in cultured stratified squamous epithelium. Subcultivated rat lingual epithelial cells grown in conventional culture demonstrate vesicles resembling MCGs (see figure 6 of ref. [5]). However, the occurrence of such granules in conventional cultures is infrequent, whereas it is common in the epithelium cultured in the lifted configuration. One possible explanation for the differences in MCG frequency may be related to the more extensive development of the spinous layer in lifted cultures. This greater development of the spinous layer may, in turn, reflect a greater time spent in transition from mitotically active basal cell to terminally differentiated keratinized cell. This greater time spent in transition might permit a greater degree of organellar development than that observed in conventional cultures. To check this possibility mitotic activity and cell transit times are currently being studied in lifted and unlifted cultures.

Differences in cell transit time and the increased development of the cell attachment specialization in lifted cultures might also contribute to the thicker and more organized keratinized cell layer observed in this type of culture. However, it appears the most important factor responsible for differences in keratinized cell layer organization observed in the two types of cultures is the positioning of the cultures with respect to the medium. Conventional cultures are submerged in medium, which can both maximally hydrate the keratinized cells as well as create shearing forces every time the culture is moved or fed. Lifted cultures are spared the shearing effect and, while humidified, the keratinized cell layer is not submerged in medium. In fact, the slight amount of medium drawn up onto the culture surface by capillary action may, by increasing surface tension, encourage tight packing of the cells. Thus, the differences in the physical environment of the two culture types would appear to be the most likely explanation for the observed differences in the keratinized cell layer.

The terminal differentiation of epidermal cells in vitro has been extensively studied by Green and his associates [29, 30, 31]. Terminally differentiated rat lingual epithelial cells in culture appear identical to the

terminally differentiated cells described by Green [31]. The cells form envelopes, they retain nuclei, and they are filled with filaments insoluble in SDS in the absence of a reducing agent. The presence of disulfide groups in the terminally differentiated cells was further confirmed histochemically. Although we have not yet electrophoretically characterized the proteins of cultured rat lingual epithelium, we presume they would demonstrate keratins as has been shown by Sun & Green [29] and Steinert & Yuspa [32].

The terminally differentiated cells in both conventional and lifted cultures appear identical. Therefore a nutrient gradient does not appear necessary for terminal differentiation [31]. However, basal feeding does appear to enhance the organization of a tissue derived from dissociated cells to a form that more closely resembles the parent tissue. In addition, lifted cultures exhibited an ongoing, synchronous terminal differentiation process in contrast to the episodic, spotty keratinization observed in conventional rat lingual epithelial subcultures. Such lifted cultures may prove useful in studying a variety of factors that influence the maintenance of a differentiated state.

Note added in proof

Fusenig has kindly called to our attention that he, too, has grown stratified squamous epithelium on raised collagen rafts and found enhanced tissue organization (Bull cancer 65 (1978) 19).

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