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FINE STRUCTURE OF SUBCULTIVATED STRATIFIED SQUAMOUS EPITHELIUM

ARNE JEPSEN,[1] DONALD K. MacCALLUM[2] and JOHN H. LILLIE[2]

[1]*Tissue Culture Laboratory, Department of Oral Pathology, Royal Dental School, Aarhus, DK 8000, Denmark, and* [2]*Department of Anatomy and Dental Research Institute, University of Michigan, Ann Arbor, MI 48109, USA*

SUMMARY

Subcultivated rat lingual epithelium derived from primary explants remains mitotically active, possesses an organellar complement similar to the parent tissue, and undergoes terminal differentiation. Successful growth of primary cultures requires an incubation temperature below 34°C and the addition of dimethyl sulfoxide (DMSO) to the medium. The subcultures retain a stable morphological phenotype through a minimum of 15 passages. Cultures are long-lived and may be maintained for one year or more in any passage.

The in vitro cultivation of cells derived from stratified squamous epithelia (epidermis; oral, esophageal or vaginal mucosae) has been the subject of numerous investigations. Short (days) and long (up to 6 months) duration cultures of cells having cytological features similar to those of the parent tissue have been established either from epithelial cells that have migrated onto a culture substratum from an explant or that have been introduced into cultures as dissociated single cells (reviews of stratified squamous epithelial cell culture are presented by Flaxman [1]; Prunieras et al. [2]; Hill & Miles [3]; Sun & Green [4]; Liu & Karasek [5]). Despite the hardiness of the native tissue type and the generally satisfactory growth of short-term primary cultures, subcultures of stratified squamous epithelium have proved exceptionally difficult to establish.

Recently, Green and his associates [6, 7, 8] have described the successful primary cultivation and subcultivation of human diploid stratified squamous epithelium and of aneuploid stratified squamous epithelium derived from a mouse teratoma. In both instances the presence of lethally irradiated fibroblasts (3T3 cells) in the cultures is essential for the growth and maturation of the epithelial cells. More recently, the successful subcultivation of rabbit or human epidermal cells through two or three passages has been reported [9, 10]. Many of the features of the parent tissue are retained in these cultures, including the ability to stratify and keratinize. Previous to these reports the only successful subcultivation of stratified squamous epithelium had been of that derived from guinea pig skin. Unfortunately, these subcultured guinea pig epithelial cells do not stratify, and they also lose cytological characteristics associated with the parent tissue, i.e., tonofibrils and desmosomes [11].

In this paper, we present observations made on subcultures of rat oral epithelium that retain many of the cytological features

of the parent tissue and which do not require the benefit of irradiated supporting cells.

MATERIALS AND METHODS

Primary cultures

Primary cultures of ventral lingual mucosa taken from 150–175 g Sprague-Dawley rats (Charles River, Wilmington, Mass.) are established in 30 cm^2 plastic culture flasks by explanting, into separate plasma clots, 5 pieces of ventral tongue, each piece being approx. 3×2×2 mm in size. The clots are allowed to form for 40 min, then the cultures are fed with a complete culture medium consisting of Minimum Essential Medium with Earle's salt mixture (MEM), 20% (vol/vol) fetal calf serum (FCS), 160 U/ml of Mycostatin (first week only) and 50 μg/ml Gentamycin sulfate or 250 U/ml of penicillin and 25 μg/ml of streptomycin sulfate. Dimethyl sulfoxide (DMSO) is added to a concentration of 0.5% (vol/vol). The cultures are gassed with 5% CO_2 in air to maintain a pH of 7.4 and incubated at 32°C.

The basic features of the primary culture system have been the subject of a brief report [12]; a comprehensive report of epithelial cell growth in primary culture is being prepared by one of us (A. J.). It is our experience in both Aarhus and Ann Arbor that the successful growth of rat oral epithelium in primary culture requires: (1) a temperature not exceeding 34°C; (2) selected FCS at a concentration of not less than 15% (vol/vol) (not all FCS will support epithelial cell growth equally well, requiring that several batches of serum be screened before selecting a particular serum lot which will yield results similar to those described in ref. [12]), and (3) the addition of 0.5% DMSO [13], (silylation grade, Pierce Chemical, Co. Rockford, Ill.).

Subcultures

When primary cultures are 20–30 days old, they are rinsed twice (30 sec each rinse) in sterile, 4°C MEM containing 0.25% trypsin (1:250; Difco Lab, Detroit, Mich.) and 5 mM Na_4EDTA (because of the calcium and magnesium in the medium, the effective EDTA concentration is 3.2 mM), pH 7.4. The cultures are then incubated at 4°C for 45–120 min in 5 ml of the MEM trypsin–EDTA mixture. The cells are dislodged from the plastic culture surface by gently scraping the surface with a silicon rubber spatula. The cells are further dispersed by drawing them through a wide bore pipette several times.

The resultant cell suspension is added to an equal volume of complete tissue culture medium and centrifuged for 4 min at 700 g. The cell pellet is dissociated by pipetting fresh medium onto the surface of the pellet and the resulting cell suspension is appropriately diluted and then added to culture flasks using the same culture medium and gassing regimen as outlined above. Successful initial subcultivation (establishing the first subculture) more often results when a 'crowding' technique is employed. This involves combining the cells derived from 3 or 4 flasks containing 20–30-day-old cultures and adding them to a single flask. Subsequent subcultivation may be carried out as described in the Results section.

Cultures are fed twice weekly with complete medium containing 0.5% DMSO. The DMSO may be omitted from subcultures; however, vacuolation, cytoplasmic retraction, and reduced cell viability have been observed in many subcultures when grown for more than 2 months in the absence of DMSO. Cultures can be kept in the same flask for periods of more than one year without subcultivation. A small percentage (~10%) of first subcultures exhibit several large (0.5 cm^2) areas of densely packed fibroblasts (see Results) which tend to become a permanent feature of the culture. Such subcultures were discarded in this study.

Drop cultures

Drop cultures are initiated by spacing 10–13 drops (~150 μl each) of culture medium (each drop containing between 100–200 cells) on the surface of a 30 cm^2 flask. One to 2 ml of sterile water is added to the end of the flask to prevent dehydration of the drops. The cultures are gassed with 5% CO_2 in air and then incubated at 32°C for 24 h. The cultures are rinsed in complete medium and then grown as outlined above.

Cell counts and cell viability

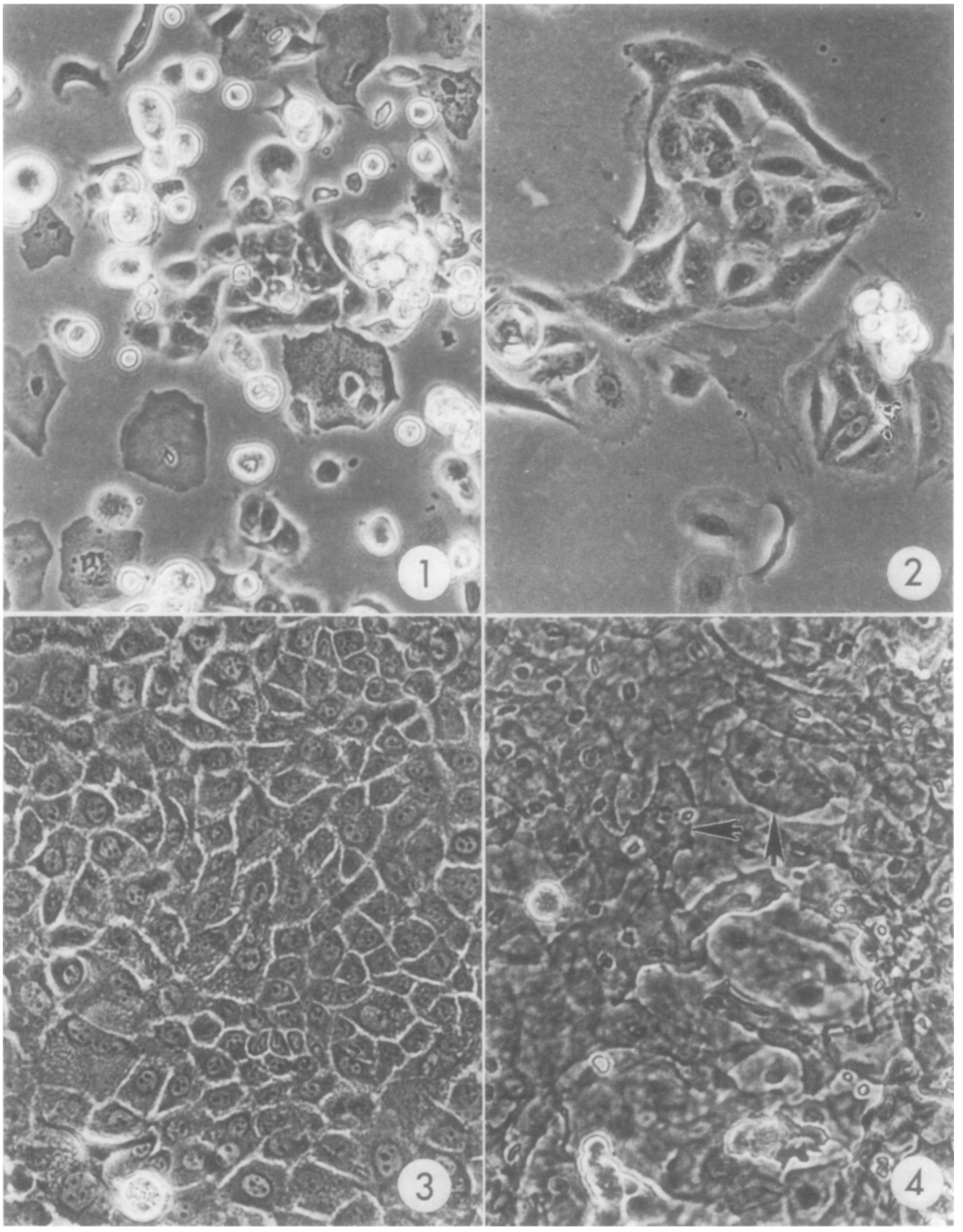
Cell counts were made using an automated cell counter (Coulter model ZF) following recommendations of the manufacturer. Subcultures 3–4 weeks old contain between 6×10^6 and 10×10^6 cells. To facilitate counting of cells in dye exclusion studies, large flakes or patches of keratinized cells (which also contain a large number of viable cells) were allowed to settle by gravity to the bottom of a centrifuge tube. The supernatant containing clusters of cells or single cells was then collected by centrifugation and thoroughly dispersed in serum-free medium. When such a procedure was employed, the resulting cell suspension contained between 5 and 10% squamoid, keratinized cells, most of which excluded trypan blue. Approx. 98% of the rounded, non-keratinized cells excluded trypan blue.

Chromosome analysis

A karyotypic analysis of cells in a fourth subculture was performed by the American Type Culture Collection (ATCC) (Rockville, Md). The analysis revealed the cells to be aneuploid with a modal chromosome number of 40 (rat $2n=42$) with 10% of the 50 cells analysed being polyploid. The cells analysed were morphologically indistinguishable from those depicted in this study.

Reimplantation of subcultivated cells:

Cells in the seventh and eighth passage were suspended in 0.5–1 ml of MEM and injected subcutaneously into 75 g outbred male Sprague-Dawley rats. Approx. 1×10^6 cells were injected in two sites. Animals were killed 1, 3 and 6 weeks following injection.



Figs 1–4. Phase contrast photomicrographs of epithelial cells subcultured at a density of 1×10^6 cells/30 cm^2 flask. ×260.

Fig. 1. Eight hours following subcultivation many viable cells have attached and spread onto the flask surface. The large keratinized cells do not attach.

Fig. 2. Three days following subcultivation most of the cells are arranged in small clusters.

Fig. 3. Eight days following subcultivation confluent cultures are characterized by closely packed cells having a high nucleo-cytoplasmic ratio.

Fig. 4. Lightly keratinized area photographed with the mature cells (*arrows*) in the plane of focus. All keratinized cells have apparently retained their nuclei.

The injection sites were processed for morphological study as described below.

Observations

Living cultures were viewed and photographed using a Zeiss UPL inverted phase contrast microscope. Ultrastructural observations were made on cultures fixed for 2 h at 4°C in a 1 : 1 mixture of 2.5% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.4, containing 2 mM $CaCl_2$ and 2% aqueous OsO_4. Some cultures were subsequently stained en bloc with 2% uranyl acetate [14]. Cultures were dehydrated in ascending concentrations of ethyl alcohol and infiltrated with a mixture of one part 100% ethyl alcohol and one part Epon-Araldite [15]. Finally, the cultures were infiltrated with several changes of pure, catalysed resin over a 48 h period and then embedded in situ with a 1–2 mm thick layer of resin present above the cells. The preparations were polymerized for 48 h at 60°C, allowed to return to room temperature, then small squares of the resin-coated flask were cut out, mounted in a microtome chuck, and sectioned with a diamond knife. Sections were collected on carbon-reinforced Formvar-coated grids and stained with uranyl acetate and lead citrate. Sections were viewed and photographed in a Philips EM 300 electron microscope.

RESULTS AND OBSERVATIONS

Phase contrast observations

When cells are inoculated in large numbers (1.25×10^6 cells/30 cm^2 flask, which represents 1/8 of the total cells in one confluent 30 cm^2 flask), they occupy approx. 50% of the culture surface. The viable, rounded cells attach to the flask surface and begin to spread onto it within 3 h following inoculation. The non-viable, flattened, partially keratinized cells do not attach (fig. 1). Within 72 h the majority of epithelial cells have spread out. The cells are arranged in clusters and present various appearances (fig. 2). Within 8 days the cultures are confluent. In the confluent cultures the cells are tightly packed, polygonal in shape, and variable in size. Tonofibrillar networks and spinous projections can occasionally be recognized (fig. 3). Although these cultures appear to be monolayers, ultrastructural study of areas similar to that depicted in fig. 3 frequently revealed 2 or 3 cell layers.

A series of changes then begins to occur in the cultures, the most conspicuous of which is the appearance of macroscopically visible opalescent areas ranging from 1 mm^2 to several cm^2. When opalescent areas are examined by phase contrast microscopy, the culture surface is covered by large squamous cells, most of which contain a pycnotic nucleus (fig. 4). (The maturation of surface cells in vitro does not completely resemble in vivo keratinization [8]). The opalescent areas which represent regions of keratinization are a continuing though episodic feature of the cultures. Opalescent regions may remain so for a period of weeks. However, the surface cells are subsequently shed and the opalescence lost. Small nucleated cells which had been obscured by the keratinized surface cells can then be viewed microscopically. A culture older than one month will usually have areas similar to those depicted in figs 3 and 4, although the opalescent areas will usually predominate. When cells are inoculated in very large numbers (5×10^6 cells/30 cm^2 flask), the cultures become confluent in 2 days, and patches of keratinized cells become obvious as early as the third day.

When cells are inoculated in relatively low numbers (8×10^3 cells/30 cm^2 flask), growth to confluence takes 4–6 weeks. The cells possess abundant cytoplasm resembling recently subcultured cells (fig. 2) until confluence is reached. The cultures then enter the maturation process described above and in a period of 8–12 weeks are indistinguishable from subcultures initiated with greater numbers of cells. Cell colonies established by drops containing 100–200 cells recapitulate the morphological stages of subcultivation described above. The colonies expand radially and will eventually become confluent (10 colonies/30 cm^2 flask). Fibroblasts (see below) were not

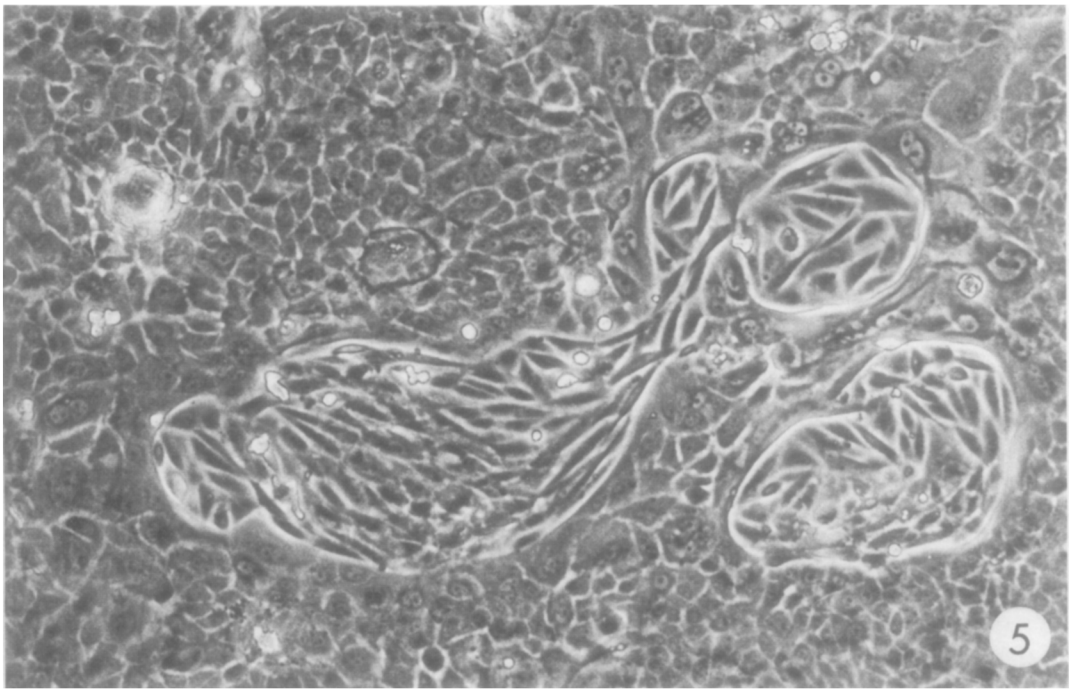


Fig. 5. Fibroblastic foci observed 10 days following a third passage subculture. Fibroblasts are apparently excavated from the culture surface by the advancing epithelial cells. These fibroblastic foci were not apparent 3 days later. Fibroblastic foci did not reappear during subsequent subcultivations of the culture depicted here. ×150.

recognized in either the low cell density or drop cultures initiated with cells from 5th or 6th subcultures.

The primary cultures used to establish subcultures always contain some fibroblasts. Occasionally a number of fibroblasts (the exact number is under investigation) sufficient to establish a visible fibroblastic component in the subculture will be carried over into the subculture from the primary culture. The presence of these fibroblasts can be recognized by the appearance of small foci containing spindle-shaped cells (which have abundant rough endoplasmic reticulum and lack a tonofilament–desmosome apparatus) surrounded by epithelial cells (fig. 5). The presence of these fibroblastic foci is short-lived in subcultures for they are rapidly displaced by epithelial cells. The area depicted in fig. 5 could not be detected 3 days later. Fibroblastic foci are rarely found beyond the 4th subculture.

Ultrastructural observations

Ultrastructural studies of rat oral epithelial cells in primary culture have been presented [16, 17]. The morphology of cells occupying a non-keratinized area in a subculture is depicted in fig. 6. The cells are multilayered, with those cells nearer the culture surface appearing more flattened than those cells attached to the culture substratum. All cells contain numerous free ribosomes, a few cisternae of rough endoplasmic reticulum, tonofilaments, and desmosomes (figs 6–8). Most cells show the presence of a Golgi apparatus that is well developed in comparison with native cells

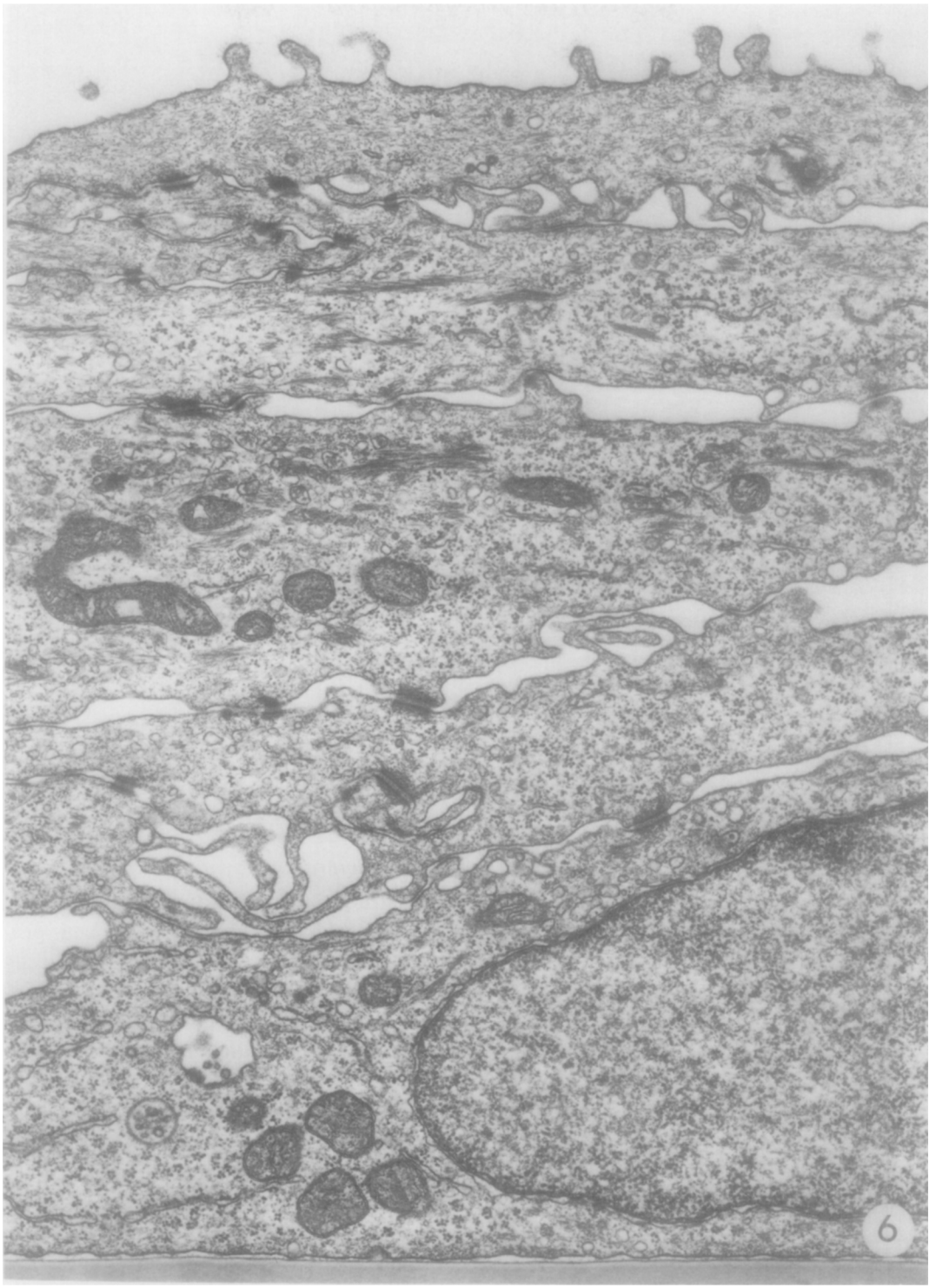


Fig. 6. Electron micrograph of a non-keratinized area in a fourth subculture. The cells are multilayered and possess tonofilament bundles and desmosomes. ×21 600.

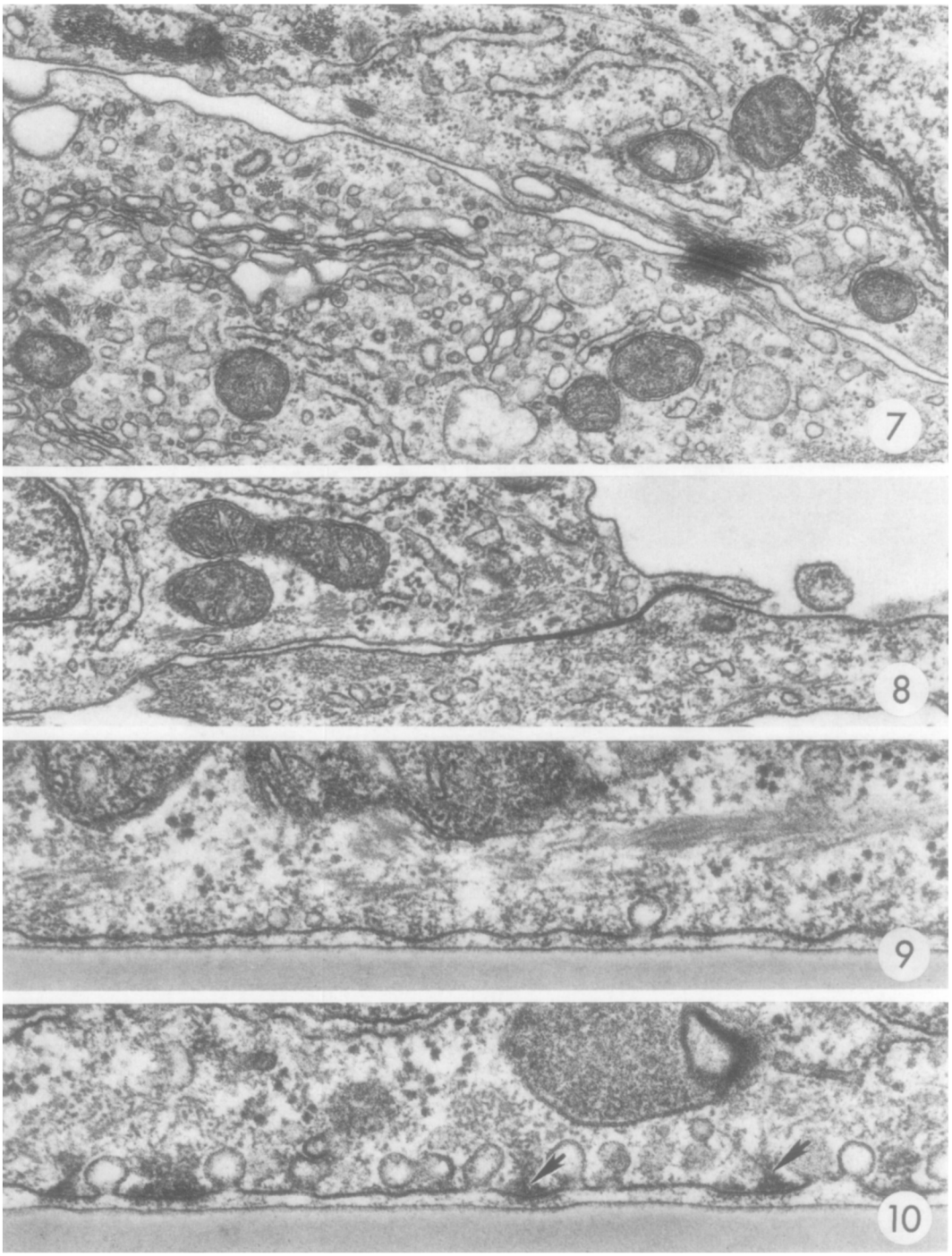
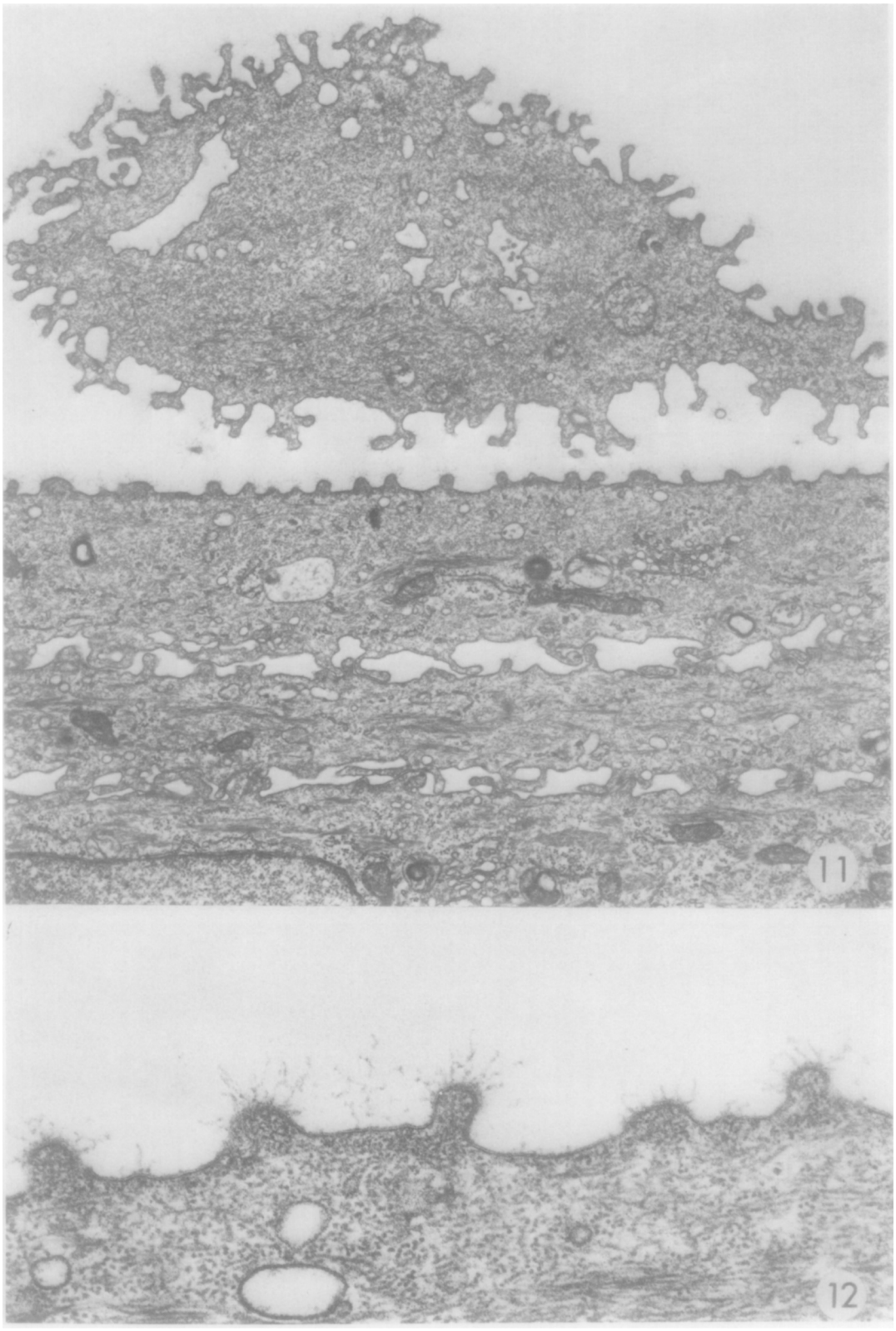


Fig. 7. The Golgi apparatus of one suprabasal cell is depicted. Cultured epithelial cells display more prominent Golgi apparatuses than do native (in vivo) cells. ×30 000.

Fig. 8. Gap junction between two cultured epithelial cells. ×65 000.

Fig. 9. Junction of an epithelial cell with the plastic culture substratum. A narrow space (10–25 nm) filled with amorphous material separates the undersurfaces of the cell from the pronounced, electron-opaque surface of the plastic. ×78 000.

Fig. 10. Hemidesmosomes (*arrows*) are occasionally encountered on the undersurface of basal cells. ×78 000.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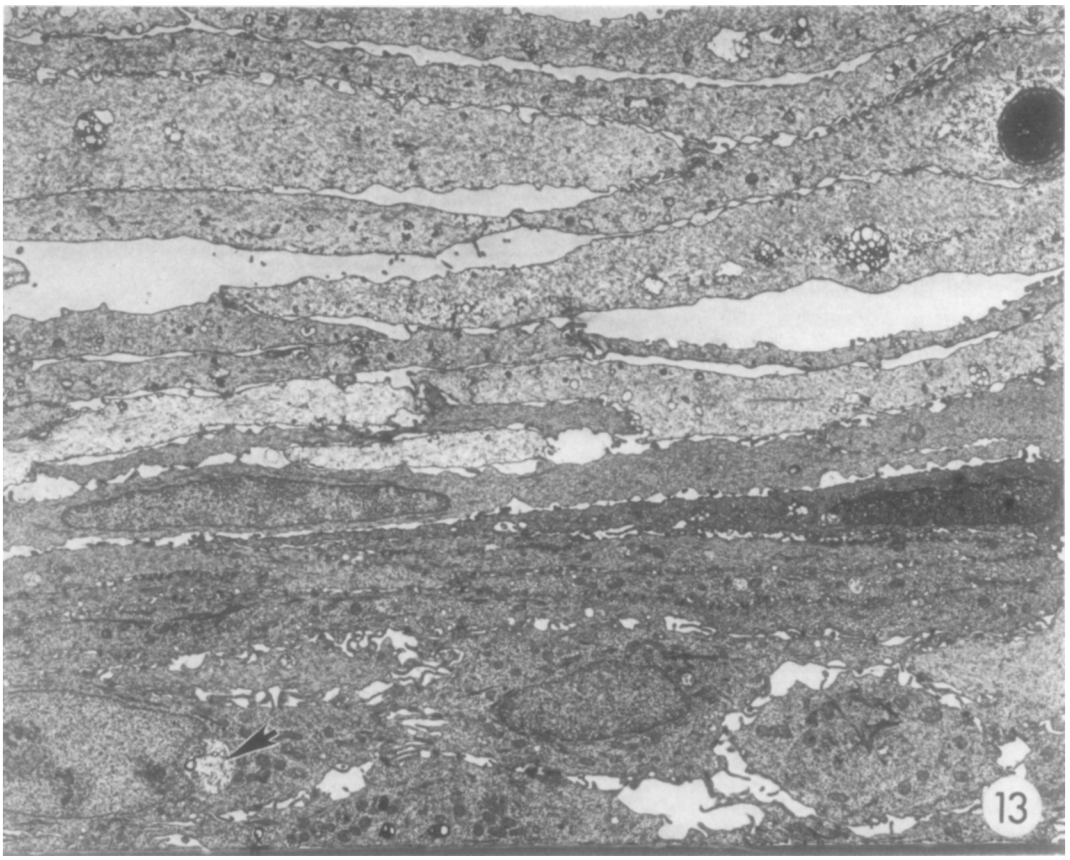

Fig. 13. Survey electron micrograph of a moderately keratinized area. A pycnotic nucleus and degenerating mitochondria are present in the keratinized cells. Focal cytoplasmic degeneration in a spinous cell is indicated by the arrow. Viable cells located beneath heavily keratinized areas occasionally demonstrated such focal degeneration. The elongate shape of the basal and suprabasal cells and the occasional widened intercellular spaces are typical of keratinized areas. ×4 000.

(fig: 7). Multivesicular bodies and secondary lysosomes are also present in the cells. Gap junctions are observed (fig. 8) in subcultured cells; however, these junctions occur less frequently than in the parent tissue. An amorphous, moderately electron-opaque material usually is present in the space between the plastic culture substratum and the undersurface of the most basal cell. The cell membrane in the region where the cell contacts the substratum usually presents a gently undulating profile and is interrupted at intervals by calveolar invaginations (fig. 9). Alternatively, and much less frequently, hemidesmosomes having both attachment plaques and peripheral densities are observed (fig. 10).

The number of cells that undergo some degree of terminal differentiation is highly variable. This variability is undoubtedly

Fig. 11. Disjunctive epithelial cell being sloughed into the culture medium. In contrast to more mature keratinized cells, intact organelles are frequently encountered while subplasmalemmal envelopes are not. ×13 500.

Fig. 12. Detail of microvilli on the surface of a superficial cell. Strands of material radiate into the extracellular space from the surfaces of microvilli but are absent from the intervening regions. ×78 000.

due to the fact that the process responsible for cell maturation is episodic and a specific region of a subculture will present different appearances depending upon the 'stage' it is in. An area in which only a single sloughing or disjunctive cell is present is depicted in fig. 11. The cytoplasm of the sloughing cell is typified by tonofilaments which are dispersed throughout the cytoplasm and by degenerating organelles, the most conspicuous of which are mitochondria (fig. 11). Cells immediately beneath disjunctive cells also present some unique cytoplasmic features.

The most obvious of these features are the dispersal of tonofilaments in the upper portions of the cytoplasm and the presence of numerous, knob-like projections on the free surface (fig. 12). Strands of an electron-opaque material emanate from the surface of these projections and appear to be limited to them. Surface projections of uppermost cells are only found in non-keratinizing areas in which the uppermost cells lack a subplasmalemmal envelope—a cytological structure found only in terminally differentiated cells [8]. An area containing many terminally differentiated cells is shown in fig. 13. These cells are also typified by tonofibrils distributed throughout the cytoplasm and by the presence of degenerating cell organelles. Pycnotic nuclei are encountered in the mature cells, which can account for up to 30 cell layers in markedly opalescent areas of a culture. The keratinized cells are morphologically identical to those observed in basally fed cultures and are further described in an accompanying paper.

Re-implantation studies

Subcutaneous injection of cultured cells into rats resulted in the formation within one week of multiple, freely movable 1–2 mm nodules. Microscopically, cystic nodules containing partially keratinized, degenerating epithelial cells were surrounded by several (5–10) layers of concentrically arranged fibroblasts with very few polymorphonuclear leukocytes, lymphocytes or macrophages present in the connective tissue. Nodules could not be found 30 days following injection.

DISCUSSION

Ultrastructural study of primary [18, 19] or subcultivated [10, 20] epithelial cells indicates the degree to which the cultured cells have retained the phenotype of the parent tissue, stratified squamous epithelium. In our study, ultrastructural examination reveals that subcultivated epithelial cells possess an organellar complement characteristic of the native tissue [21, 22, 23] and that the cells both stratify and mature. There are differences between the in vitro organization of stratified squamous epithelium and the parent tissue. The more conspicuous differences include lack of keratohyalin and membrane-coating granules, a reduced complexity of the tonofilament–desmosome apparatus, and a more squamoid appearance of the basal cells.

Despite the absence of some organelles and inclusions that are associated with cellular maturation in the parent tissue, the cultured cells do undergo terminal differentiation. (The terminal differentiation of rat lingual epithelial cells in subculture as compared with the terminal differentiation of stratified squamous epithelium in other culture systems is more fully discussed in an accompanying paper [35]. Exfoliation of cells also occurs in regions not exhibiting the terminal differentiation process. The exfoliated cells resemble those described in non-keratinized areas of the oral region, as

do the superficial cells which possess prominent microvilli (probably two-dimensional profiles of microplicae [24]), each having a conspicuous extracellular coating [25, 26]. A similar conformation of coated microvilli is seen in a primitive epithelium, embryonic periderm [27]. In fact, the decreased number of desmosomes and tonofibrils together with the reduced electron opacity of the ground cytoplasm all contribute to a more embryonic [27] than mature [21, 26] appearance of the cultured epithelium. Culture conditions are also apparently responsible for the reduced numbers of hemidesmosomes observed in this study. The occurrence of these attachment specializations in cultured stratified squamous epithelium has been noted in only two other studies, both of which described epithelial outgrowth in primary culture [28, 29].

A series of disparate techniques for successfully subculturing a stratifying, keratinizing epithelium appears to be emerging. One technique employs exogenous supporting cells [7], another employs of trypsin–EDTA dissociation [30] and conventional culture techniques [10], and a third employs trypsin–EDTA dissociation technique, a collagenous substratum, and different media to affect non-proliferative or proliferative cultures [9]. The technique described in this paper used trypsin–EDTA dissociation, 0.5% DMSO in the culture medium, a lower (32°C) than conventional incubation temperature, and a high initial cell inoculum. Each technique appears to yield cells that while closely resembling those of the parent tissue are not completely identical to it. Considerably more work will be necessary before those factors necessary for successful subcultivation of stratified squamous epithelium are fully understood.

Two features of the technique used in this study appear unique and warrant further comment. Either primary cultures or subcultures will degenerate within 6 weeks if grown at 37°C. A recent, independent study [31] of dissociated mouse epidermal cells in primary culture has confirmed the temperature sensitivity of at least rat and mouse stratified squamous epithelium in culture. DMSO has a bewildering set of effects on cells in culture [32]. In the system described here, we believe DMSO may act as a carrier for some essential molecule(s) through the keratinized layer of cells, which can occupy nearly the entire surface of the cultures. This belief is based upon the following observations: (1) The viable cells located beneath heavily keratinized areas frequently show signs of autolysis, while cells in non-keratinized or lightly keratinized areas of the same culture do not. (2) Epithelium grows and differentiates to a high degree in the absence of DMSO when fed from beneath as described in an accompanying paper [35].

Karyotypic analysis of one subculture indicated aneuploidy. We have not had an opportunity to conduct the comprehensive cytogenetic analyses required to establish whether or not the chromosome number or morphology of the cultures remains stable. Subcutaneous injection of cultured cells into outbred Sprague-Dawley rats resulted in cyst formation and subsequent degeneration. Neither signs of invasiveness nor tissue rejection were observed. The formation of cysts filled with partially keratinized cells following subcutaneous injection is similar to the behavior of non-malignant epithelial cells which become separated from a surface [33]. Based upon that behavior, and pending further study, we believe the cells are not malignant.

The presence of fibroblasts in outgrowths from primary explants of rat tongue occurs

with a frequency of almost 100%, and at least some of these fibroblasts are carried over into initial subcultures. The fibroblasts can be recognized as discrete cellular foci surrounded by the sheet of epithelium. Additionally, fibroblasts may be recognized by initiating drop cultures of the type described above. Apparently fibroblasts released from the culture surface reattach and begin to divide, whereas epithelial cells do not. Using drop cultures, we have detected fibroblasts in cultures which appeared to be entirely epithelial in nature and which contained no aminopeptidase positive cells [34].

Many of our cultures (including those used for this study) do not show any fibroblasts when the drop culture technique is employed. However, in view of the substantial evidence [4] that at least some fibroblastic contribution is necessary for epithelial cell growth in vitro, we prefer to regard our cultures as fibroblast-poor rather than fibroblast-free. Subcultivated lingual epithelium does not, however, require exogenous supporting fibroblasts to remain mitotically active and phenotypically similar to the tissue from which it is derived. An even greater degree of differentiation of subcultivated lingual epithelial cells can be achieved when the cells are grown on a collagenous substratum in organ cultures as described in the following paper.

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