

# Involucrin Synthesis and Tissue Assembly by Keratinocytes in Natural and Cultured Human Epithelia

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**ABSTRACT** Different stratified squamous epithelia, whether they bear a stratum corneum or not, are shown by immunofluorescence to possess the precursor protein of the cross-linked envelope that is characteristic of epidermal s. corneum. This protein, involucrin, is not present in the deepest epithelial cells but appears in the course of their outward migration. The boundary at which involucrin first appears can sometimes be correlated with a visible boundary between zones of large and small cells.

Cultured keratinocytes, derived from all stratified squamous epithelia (epidermal, corneal, conjunctival, esophageal, lingual, and vaginal), form colonies that grow together to form a stratified epithelium. The cells of the basal layer are nearly always free of detectable involucrin, but, in contrast to the natural epithelium, this protein usually makes its appearance in the cells immediately above the basal layer.

When a cultured epithelium derived from epidermal keratinocytes is detached and applied as a graft to animals, the cells flatten and the distinctness of the basal layer is at first reduced; but with time the organization of the epithelium becomes more characteristic of epidermis. Cell size and shape become more orderly along the cell migration pathway, and involucrin first appears at some distance from the basal layer, instead of in immediately suprabasal cells, as in the cultured epithelium. The progeny of dissociated and cultured keratinocytes are therefore able, when grafted, to reassemble an epidermis in which the timing of specific gene expression is restored to that of the original tissue.

Most cell types cultivated on glass or plastic surfaces form only rudiments of the tissue structure in which they ordinarily reside. Perhaps because their normal function is to cover a surface, epithelial cells may be able to form a tissue structure in surface culture more effectively than other cell types. Cultivated epidermal cells generate a stratified epithelium whose cells are linked by typical desmosomes. The natural polarity of the epithelium is recreated, in that the basal layer of the culture possesses all the cells capable of multiplication (12). As the cells move from basal layer outward, they undergo certain features of terminal differentiation. As in natural epithelia (11, 18, 24), the cells enlarge (20). Eventually they become squame-like and develop a protein envelope cross-linked by cellular transglutaminase (5, 15, 20). The squames usually do not form

an adherent layer of anucleate stratum corneum, but they do digest their nuclei and cytoplasmic organelles after they detach from the epithelial surface (5).

In what follows, we compare different stratified squamous epithelia and cultured epithelia derived from them in regard to the biosynthesis of involucrin (22), a protein precursor of the cross-linked envelope that is a prominent component of s. corneum (15, 16). We find that all stratified squamous epithelia make this protein whether or not they possess a s. corneum. As in the case of epidermis (16), involucrin is not found in the deepest layers of the epithelia but makes its appearance only in the course of the outward migration of the cells. Involucrin synthesis takes place in cultured keratinocytes, but its regulation is not precisely that in the corresponding natural epithe-

lium. When a cultured epithelium derived from epidermal keratinocytes is grafted onto an animal, involucrin synthesis becomes regulated more precisely.

## MATERIALS AND METHODS

### Human Tissues

Through the kindness of Drs. R. McClusky and R. Taft and Mr. Joseph Stukas, epithelial tissues were obtained from autopsies performed at the Massachusetts General Hospital, within 6–24 h after death. Tissues for histological examination were either frozen in isopentane cooled in liquid nitrogen, and stored at  $-70^{\circ}\text{C}$ , or were fixed in 3.7% formaldehyde in isotonic buffer containing 0.01 M phosphate, pH 7.3. Tissues for culturing were placed in medium containing 20% fetal calf serum and kept at  $4^{\circ}\text{C}$ .

### Cell Culture

Keratinocyte cultures were grown from epidermis (13) or other stratified squamous epithelia (21). Cultures were easily derived from tissues taken up to 24

h after death. Tracheal keratinocytes were derived from a surgical specimen. The tissue was washed several times with isotonic phosphate buffer. Most of the connective tissue was dissected away, and the epithelium was minced thoroughly. The mince was then placed in a trypsinizing flask and disaggregated with 0.25% trypsin and 0.005% EDTA. The suspended cells were centrifuged and inoculated at different densities into 60-mm dishes containing a supporting layer of  $5 \times 10^6$  irradiated 3T3 cells (13). The cultures were fed with the Dulbecco-Vogt modification of Eagle's medium containing 20% fetal calf serum, hydrocortisone at  $0.4 \mu\text{g}/\text{ml}$ , and  $10^{-10}$  M cholera toxin (6). Beginning 2–3 d later, epidermal growth factor (EGF) purified according to Savage and Cohen (19) was added to the culture medium at 10 ng/ml (14). Cultures were refed every 3–4 d and grown to confluence.

### Preparation of Tissue Samples for Immunofluorescent Staining

Frozen tissues taken directly from the donor were embedded in Tissue Tek (Fisher Scientific Co., Pittsburgh, Penn.) and  $8\text{-}\mu\text{m}$  sections were cut in a cryostat and stored at  $-70^{\circ}\text{C}$ . Before staining, the sections were air-dried for 1 h at room temperature. Tissues fixed in formaldehyde were dehydrated, embedded in

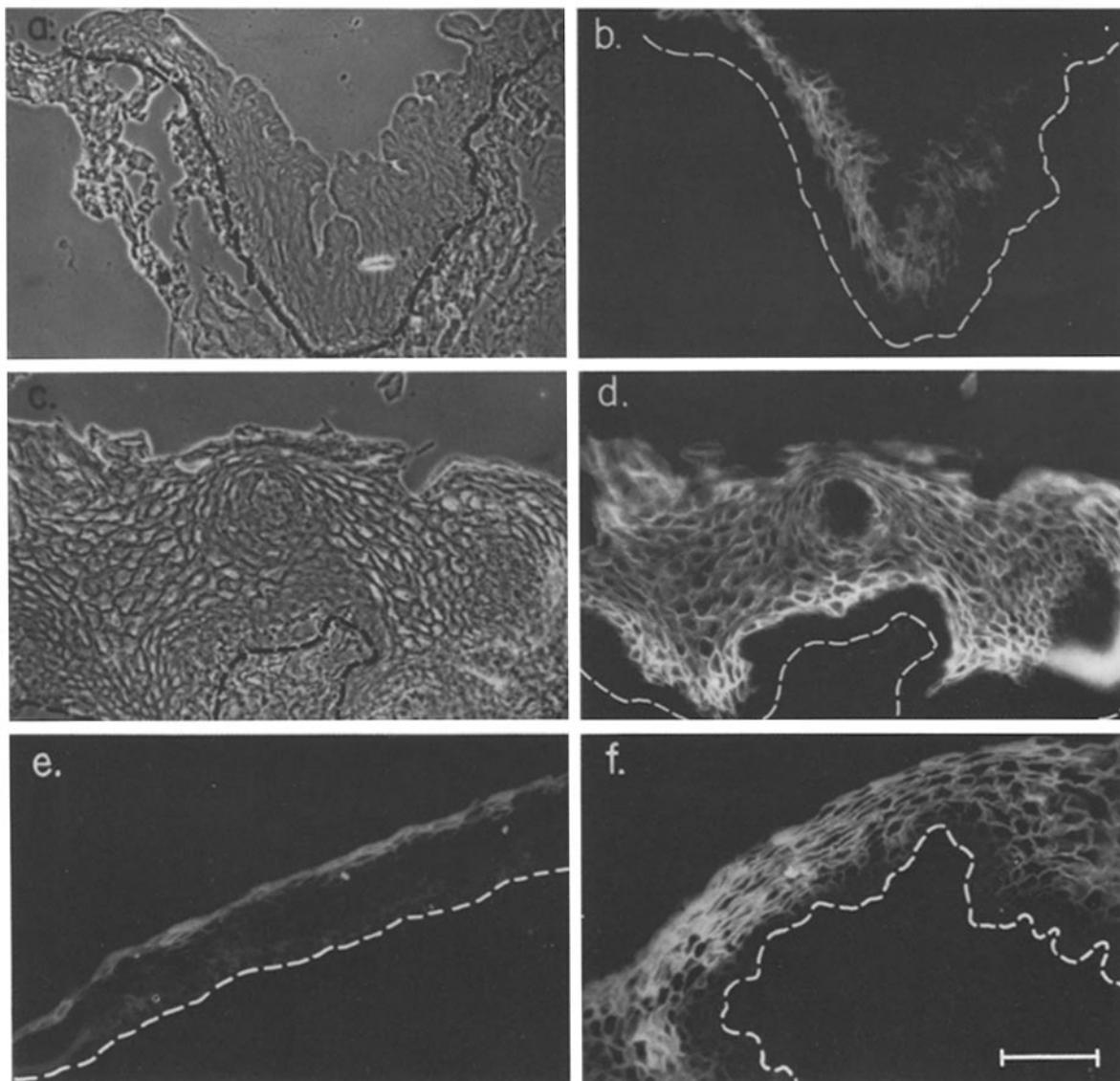


FIGURE 1 Distribution of involucrin in three stratified squamous epithelia examined in frozen section. Conjunctival epithelium: a, by phase-contrast microscopy; and b, stained by immunofluorescence using antiserum to involucrin. Lingual epithelium: c, by phase microscopy; and d, stained with antiserum to involucrin. e and f, corneal epithelium stained with antiserum to involucrin, showing extremes in depth of the zone containing involucrin. Note that in all three epithelia the deepest cell layers do not possess involucrin; any faint staining in this region was no greater than that produced by preimmune serum. In the more superficial layers the involucrin is concentrated at the cell periphery. Hatched lines indicate the position of the basement membrane. Bar,  $100 \mu\text{M}$ .

paraffin, and sectioned. The sections were then rehydrated for staining. Cultured epithelia were detached, using the enzyme Dispase II (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) at 1.2 U/ml of serum-free medium (7). The epithelia were then rinsed with isotonic buffer and floated over a round Whatman filter paper (Whatman, Inc., Clifton, N. J.) 2 cm in diameter; the buffer was then gently aspirated. The epithelium was spread out over the filter paper, the edges were tucked under, and the sample was air-dried briefly to anchor the epithelium to the paper. The preparation was then fixed in formaldehyde and prepared for sectioning and staining in the same way as the natural epithelia.

### Immunofluorescent Staining

Sections of human cornea, conjunctiva, esophagus, tongue, vagina, trachea, and bladder and the cultured epithelia derived from most of these tissues were stained with antiserum to involucrin (16) using indirect immunofluorescence. Briefly, 20  $\mu$ l of a 1:20 dilution of the rabbit antiserum were added to frozen or fixed and rehydrated sections of the various tissues and cultured cell sheets, and the preparations were incubated at 37°C for 45 min. The sections were then carried through three consecutive washes of 5 min each, using isotonic phosphate buffer. 20  $\mu$ l of diluted FITC-conjugated goat anti-rabbit immunoglobulin (Miles Biochemicals, Elkhart, Ind.) were added to the sections, and the preparations were incubated again at 37°C for 45 min. After three washes of 5 min each, a cover slip was mounted over the section using gelvatol (Monsanto Co., St. Louis, Mo.) (17). The sections were examined in a Universal Zeiss Photoscope using epifluorescence. Photographs were taken with Ektachrome 400 daylight film. The specificity of all positive reactions was controlled by the use of preimmune serum.

### Grafting

The procedure used in grafting cultured human epithelia to athymic mice has been described (2).

## RESULTS

### Presence of Involucrin in Different Stratified Squamous Epithelia

Frozen sections of human conjunctiva, cornea, and tongue were cut on a cryostat and stained with antiserum to involucrin. Although they lacked a s. corneum, these epithelia were stained by the antiserum (Fig. 1). As was found earlier for epidermis (16), no involucrin was present in the deepest cell layers, including the basal layer; this protein generally appeared not less than three to four cell layers above the basement membrane. Also, as in the epidermis, involucrin was not distributed uniformly in the cell cytoplasm but became concentrated peripherally before the time of cross-linking. Frozen sections of esophageal and vaginal epithelium showed the same features of involucrin distribution as the epithelia illustrated in Fig. 1. Although the point cannot be settled by light microscopy of sections, involucrin is on the cytoplasmic side of the cell membrane, because it remains within the cells after trypsinization (22) and the cross-linked envelope to which it gives rise is intracellular (5).

Formalin fixation suppresses involucrin staining of the cross-linked envelopes of s. corneum, most likely by preventing penetration of the antiserum. Formalin fixation also reduces the staining of the peripheral uncross-linked involucrin in living cells and makes the staining more diffuse. This accounts for the difference in staining between Fig. 1 and other figures. Because the formalin fixation makes the tissue easier to process, we used it for experiments whose aim was solely to identify those living cells containing it.

As in frozen sections, esophageal and vaginal epithelium showed strong staining (Fig. 2). In the vaginal epithelium, involucrin-containing cells extended to within a few cell layers above the basal layer, but in the esophageal epithelium the first cells containing involucrin were located at a considerably greater distance from the basal layer, as in the epidermis. In

both epithelia, a boundary could be identified under phase-contrast microscopy (*c* and *d*) between zones of large and small cell size, the large cells possessing much clearer cell borders. When this boundary was transposed to the image produced by immunofluorescence, it corresponded closely to the boundary between involucrin-positive and -negative regions (Fig. 2 *b* and *d*). The same relation could be seen in lingual epithelium (Fig. 1). In other stratified squamous epithelia, the increase in cell size was sufficiently gradual that no such boundary could be observed in the unstained sections.

### Presence of Involucrin in Cultured Keratinocytes Derived from Different Epithelia

Keratinocytes were grown from disaggregated epithelial cells of skin, cornea, conjunctiva, esophagus, tongue, vagina, and trachea. Colonies formed and grew together to form a confluent epithelium. The epithelium was fixed, embedded, sectioned, and stained. The cultured epithelium prepared from epidermal keratinocytes contained more than five cell layers, each being flattened and extended (Fig. 3 *a*). When the epithelium was stained, involucrin was evident through most of the thickness of the epithelium (Fig. 3 *b*); only the basal cells seemed to lack

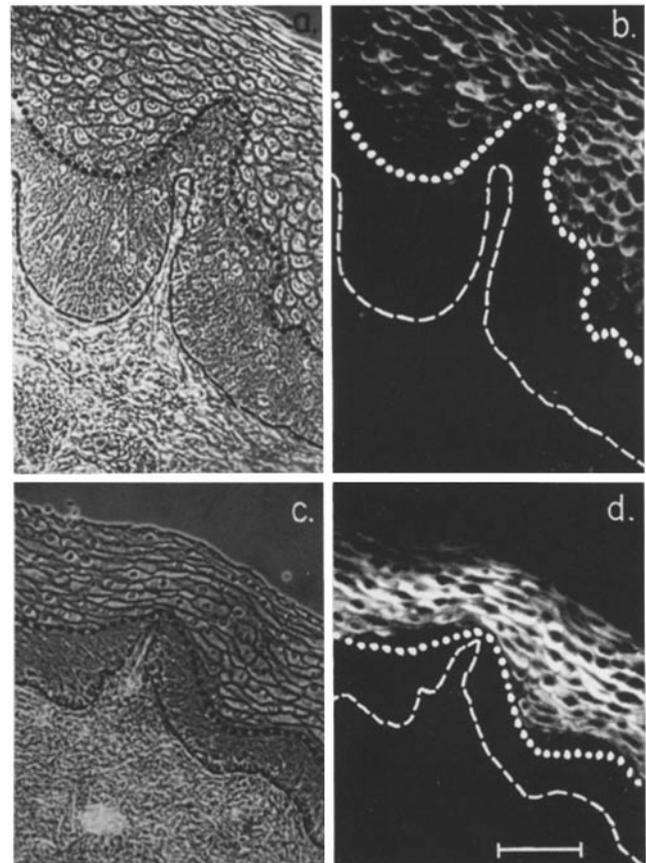


FIGURE 2 Distribution of involucrin in two stratified squamous epithelia examined in formaldehyde-fixed sections. Esophageal epithelium (*a* and *b*) and vaginal epithelium (*c* and *d*). *a* and *c*, phase-contrast microscopy; *b* and *d*, same sections stained with antiserum to involucrin. Hatched line indicates position of basement membrane, and dotted line indicates boundary between small cell and large cell zones of the epithelia. In this and subsequent figures, the formaldehyde fixation masks the peripheral staining of involucrin revealed in unfixed sections (cf Fig. 1). Bar, 100  $\mu$ M.

it, but resolution was poor because of the extreme flattening of the cells. The resolution was much improved by removing the living epithelium from the dish before it was fixed. This was accomplished using Dispase, an enzyme that detaches the entire epithelium without affecting the junctions between cells (7). The epithelium is elastic and, as it detaches, it shrinks to about half the diameter or one quarter of the area, thereby becoming about four times thicker (Fig. 3 *c*). In the process, the shape of the cells also changes. This was particularly true of the basal cells, which became columnar, as they are in the native epithelium. When stained by immunofluorescence, the detached epithelium was seen to contain the involucrin in the cells above the basal layer (Fig. 3 *d*), but the now much thicker basal layer was clearly devoid of this protein.

Cultured epithelia derived from other stratified squamous epithelia showed only small variations from this pattern. Those prepared from conjunctival, corneal, esophageal, and vaginal keratinocytes all contained cells possessing involucrin (Fig. 4). The same was true of cultures of lingual keratinocytes. The cells containing involucrin were, with infrequent exception, all suprabasal; but, in contrast to the natural epithelium, in which synthesis began at least several cell layers above the basal, involucrin was synthesized immediately above the basal layer. There was even occasionally a stained cell occupying a basal position, but such cells extended into the suprabasal layer as well and were probably in transition from a basal to a suprabasal position (Fig. 4 *j* and *l*).

Tracheal epithelium is not a stratified squamous epithelium and by immunofluorescence, sections of this tissue did not reveal keratinocytes clearly possessing involucrin. Nevertheless, as reported earlier (8), when tracheal epithelial cells are cultivated by the method used for keratinocytes, typical keratinocyte colonies develop. These cells are rich in keratins (E. Fuchs and H. Green, unpublished results) and can assemble

cross-linked envelopes with as high frequency as epidermal keratinocytes (our unpublished results). When a cultured epithelium of keratinocytes derived from trachea was stained by immunofluorescence, the suprabasal cells were found to contain involucrin (Fig. 4 *l*).

### *Reassembly of Tissue Structure and Timing of Involucrin Synthesis after Grafting of a Cultured Epithelium*

In view of the possible application of cultured epithelia to human grafting (12), it is interesting to observe that dissociated and cultured keratinocytes can restore epidermal structure after the cultures are applied as a grafts (2, 4, 10, 23, 25).

In the period shortly after grafting, stratum granulosum and stratum corneum formed, but the organization of the cells in other respects was not typical of epidermis (Fig. 5 *a*). The cells, particularly those of the basal layer, remained flattened, and there were no rete ridges. Over a period of many weeks the architecture of the grafted epithelium became more like that of the normal epithelium: the typical shape of the cells was restored, and they enlarged progressively after they left the basal layer (Fig. 5 *b*). The basal cells became columnar and the conformation of the basement membrane became ridged. When the cells were stained with antiserum, the position at which cells began to synthesize involucrin shifted from immediately suprabasal, as it had been in the cultured epithelium, to a position characteristic of epidermis (Fig. 5 *d*).

### DISCUSSION

The outer cell layers of all stratified squamous epithelia examined were found to contain involucrin and, in unfixed tissues, the protein was seen to be concentrated peripherally

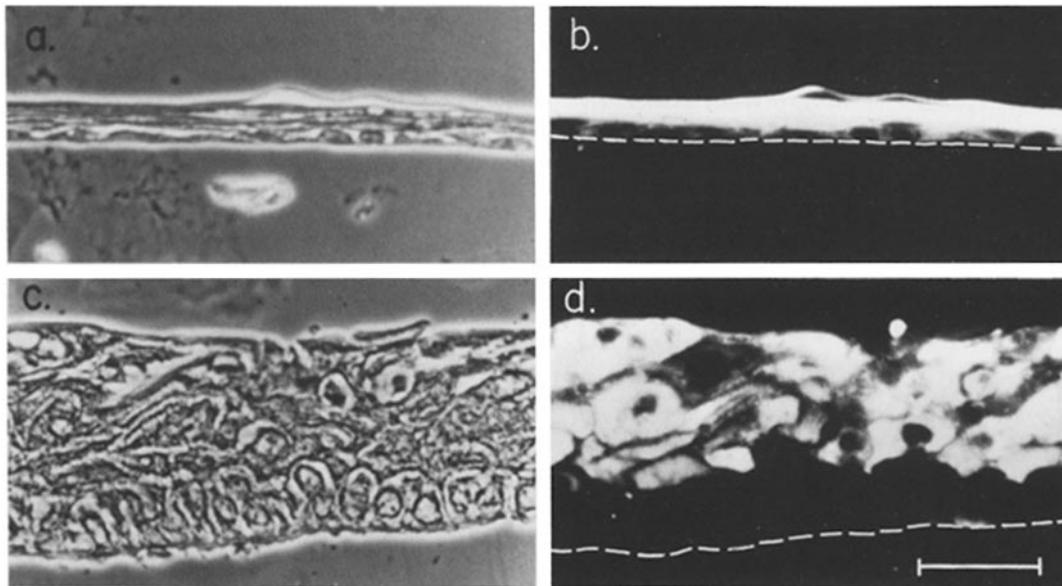
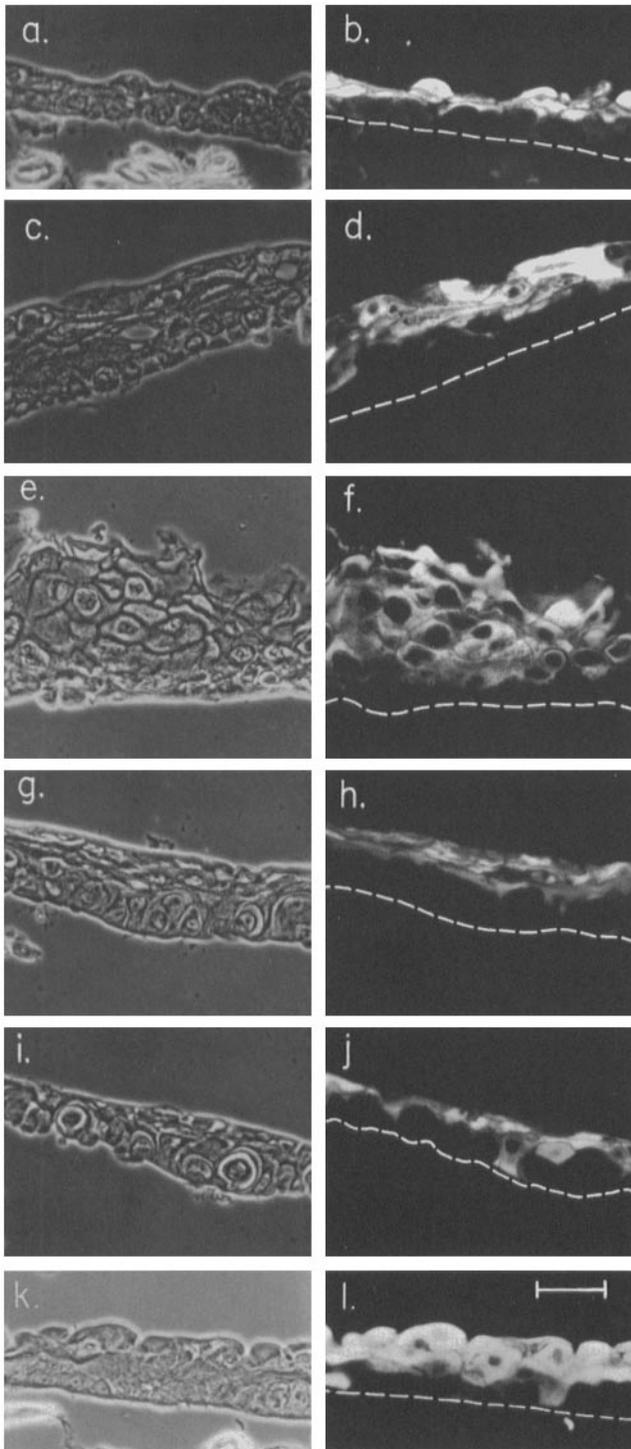


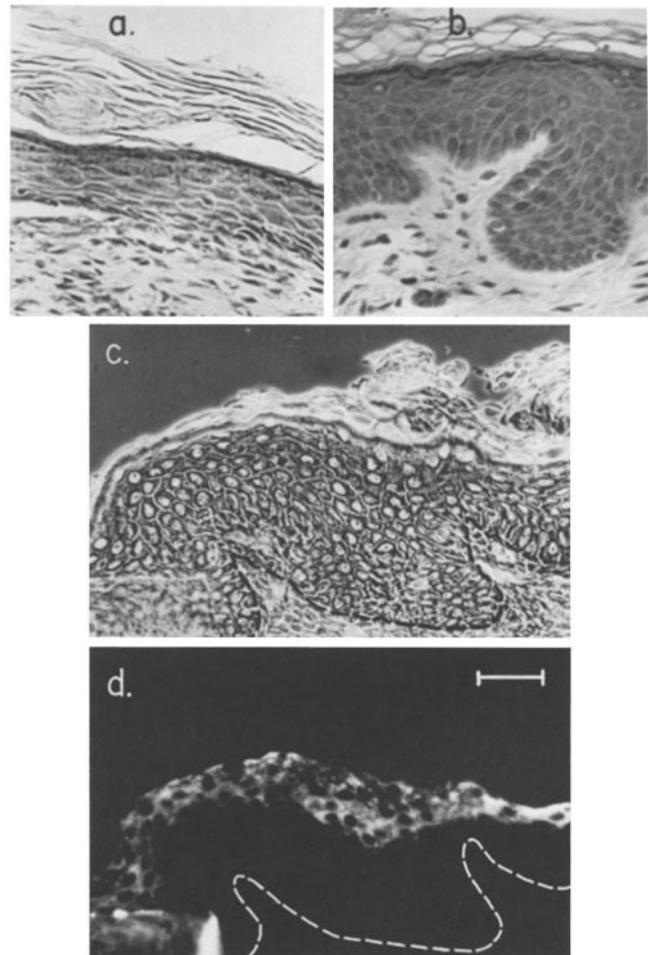
FIGURE 3 Epithelia formed by cultured epidermal cells. An epithelium was fixed in the culture vessel with formaldehyde and then removed by dissolving the plastic with xylene. The epithelium was embedded in paraffin and sectioned. Comparison of the phase-contrast image (*a*) with the fluorescent staining pattern of the same section (*b*) shows that the involucrin is present in cells down to, but not including, the basal layer. A duplicate epithelium was detached with dispase; this permits the epithelium to retract and thicken. The epithelium was then fixed with formaldehyde, embedded, and sectioned. *c* and *d* show phase-contrast and fluorescent images of the same section. Note that the basal cells have become columnar and show more clearly than in the unretracted epithelium the absence of involucrin. Bar, 50  $\mu$ M.



**FIGURE 4** Cultured epithelia formed by keratinocytes derived from different epithelia. Each epithelium was detached with Dispase before fixation. Each pair of photomicrographs shows phase-contrast and immunofluorescent images of the same section. *a* and *b*, conjunctiva; *c* and *d*, cornea; *e* and *f*, vagina; *g* and *h*, esophagus; *i* and *j*, esophagus; and *k* and *l*, trachea. In all epithelia, the basal layer is nearly always free of involucrin. Some differences in cellular organization seem to reflect the individuality of the epithelium; for example, the epithelium produced by vaginal keratinocytes (Fig. 3 *e* and *f*) seem to bear a closer resemblance to the native epithelium (Fig. 2 *c* and *d*) than do cultures of some other keratinocyte subtypes. Bar, 50  $\mu$ M.

within the cells, as it is in epidermis. Staining was less intense in the corneal and conjunctival epithelium than in others, especially after formalin fixation. Two epithelia other than stratified squamous were also examined for the presence of involucrin in both frozen and formaldehyde fixed sections; no brightly stained cells similar to keratinocytes were detected in the epithelium of either trachea or urinary bladder; but faint fluorescence made it impossible to completely exclude the presence of involucrin or cross-reacting material.

Although it does not possess a *s. corneum*, human corneal epithelium is known to possess some cells with cross-linked envelopes on its outer surface (21). We found the same to be true for esophageal epithelium. Freshly obtained tissue contained some cells with envelopes insoluble in SDS and mercap-



**FIGURE 5** Improvement of epidermal structure and control of involucrin synthesis after grafting a cultured epithelium. Epidermal keratinocytes (strain AN) were grown into confluent cultured epithelia. The epithelia were detached with Dispase and applied as full thickness grafts onto athymic mice. 10 d after grafting (*a*), an epithelium has attached, acquired a granular layer and begun to accumulate *s. corneum*, but cellular organization is still imperfect. After 108 d (*b*), the cellular organization is greatly improved; there are rete ridges, a columnar basal layer, and an orderly gradation in cell size (*a* and *b* stained with hematoxylin and eosin). Another region of the reconstituted epidermis at 108 d is shown by phase microscopy in (*c*). The gradation of cell size is again apparent. The distribution of involucrin in the same section is shown by immunofluorescence in (*d*). Bar, 50  $\mu$ M.

toethanol; and the majority of cells dissociated from the epithelium made such envelopes when they were held in stabilized suspension culture (for the method employed, see reference 5). Cultured epithelia derived from keratinocytes of any origin behaved quite similarly: they all made involucrin and, when held in suspension culture, disaggregated cells were able to assemble cross-linked envelopes.

Cultured epithelia did differ from the corresponding natural epithelia in a number of respects (see also 3): (a) Even when derived from epidermis, the cultured epithelium possessed virtually no s. granulosum or s. corneum. (b) The cells of cultured epithelia were much more flattened; this was partly corrected in the basal layer by detaching of the epithelium from the vessel surface. Because of the flattening, no indication of cell size can be obtained from sections through the epithelium. (c) Involucrin was made relatively early in the course of cell migration.

The onset of involucrin synthesis in keratinocytes as they move toward the outer surface of the epithelium is clearly a controlled process that is part of terminal differentiation. At some point, a signal must lead to the appearance of involucrin mRNA in the cells (22). The cell position at which involucrin synthesis begins is different for different stratified squamous epithelia, but that position may sometimes vary considerably in different samples of the same epithelium (Figs. 1, 2, and 5).

From the location of involucrin in the immediately supra-basal cells of the cultured epithelia, it might appear that this protein could play a role in the departure of cells from the basal layer; but cells in the natural epithelia do not contain detectable involucrin until they are considerably beyond the basal layer. Therefore it seems most reasonable to regard the earlier onset of involucrin synthesis in the cultured epithelia as due to accelerated terminal differentiation but not as the cause of cell migration.

A curious difference between the epidermis and some of the other stratified squamous epithelia is the frequent occurrence in the latter of a sharp boundary between zones of small and of large cell size (Figs. 1c and 2), only the zone of large cells possessing involucrin. It seems possible that the clarity of the cell borders under phase-contrast microscopy owes something to the presence of the involucrin, even though it is not yet cross-linked. It is difficult to see how a discontinuity in cell size could exist if both the transition of cells through the epithelium and the rate of cell enlargement were in a steady state. One possibility is that the process of cell enlargement is coordinated in a group of cells separated by a distinct boundary from a subjacent group of cells that, for the time being, remain small. Another possibility is that a group of large cells moving laterally within the epithelium could create a sharp boundary between itself and a deeper group of small cells. In culture,

keratinocytes presumably linked by desmosomes are known to move in groups (9, 20; see also reference 1).

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