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Differentiation

Retinoids alter the direction of differentiation in primary cultures of cutaneous keratinocytes

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Abstract. The effects of vitamin A on the morphological expression of differentiation were studied in cell cultures of cutaneous keratinocytes from the newborn rat. The cells were first cultivated in a medium containing 0.11 m*M* calcium until a confluent monolayer had been formed. Stratification and terminal differentiation were then triggered by raising the calcium concentration of the medium to 1.96 m*M* ('normal' culture). The rise in the concentration of calcium was coupled with the addition of retinol (RL) or retinoic acid (RAC) to the medium to produce an excess of vitamin A (high-retinoid culture). Delipidized serum was used to produce a deficiency of vitamin A (low-retinoid culture). The tissue organization and the ultrastructure of the keratinocytes in the stratified culture were the same as those seen in conventional cultures and skin explants. These stratified cultures expressed the morphological features of the epidermis of intact skin. The addition of RL or RAC to the medium enhanced features characteristic of the secretory epithelium, such as the formation of an extensive endoplasmic reticulum, an enlargement of the Golgi zone, and an increase in the number of vacuoles. At the same time, the addition of retinoids diminished features characteristic of the terminal differentiation of the stratified squamous epithelium, such as stratification and keratinization. Deficiency of vitamin A in the medium resulted in a culture with many differentiated layers. The differentiated cells of the low-retinoid cultures contained densely packed tonofilaments and synthesized products that reacted with the monoclonal antibody AE2 that is specific for keratin peptides which are markers of epidermal differentiation. In the cell culture system that is presented here, an excess of retinoids redirected epithelial differentiation from a stratifying and keratinizing epithelium towards a secretory epithelium. This system is a useful tool for elucidating the mechanisms responsible for the effect of vitamin A on the differentiation of epithelial cells.

Introduction

Retinoids, as well as vitamin A and its natural and synthetic analogs, have a major effect on the differentiation and proliferation of epithelial cells. Vitamin A is required for the normal maintenance and differentiation of epithelial tissues in vivo and in vitro [8, 22, 25]. The amount of vitamin A available modifies epidermal differentiation. An excess of the vitamin reduces the expression of terminal differentiation of stratified squamous epithelia, inhibits keratinization, and enhances the expression of secretory epithelia, sometimes resulting in mucous metaplasia [14, 18, 27, 35]. A deficiency of the vitamin results in squamous metaplasia and an excessive accumulation of keratins [5, 32].

The concentration of calcium in the medium regulates the growth and differentiation of keratinocytes in culture. Previous studies have shown that when mouse keratinocytes are cultured in medium with 0.02–0.09 m*M* calcium [16] or human keratinocytes are cultured in medium with 0.1 m*M* calcium [31], the cells proliferate and form a monolayer but do not stratify. Raising the concentration of calcium to greater than 0.1 m*M* induces the stratification and terminal differentiation of such cultures [15, 16]. However, these studies do not describe in detail the morphology and tissue organization of the cultures after the calcium concentration had been raised. Moreover, these experiments were performed using a calcium concentration in which the cultures did not survive for longer than 10–15 days [16, 36].

Both differentiated and undifferentiated populations of cells are found in primary cultures of newborn rat keratinocytes. This is a result either of cultures starting to stratify before they are confluent, or of clumps forming when basal cells are suspended in the culture medium. When these clumps are plated, the cells that attach to the substratum become part of the basal layer, while others remain as mounds on top of the culture. The cells in these mounds may partially differentiate or autolyze and, thus, contribute further to the diversity of the culture [6].

Cultivating the cells in a medium with a low concentration of calcium until a confluent monolayer is formed and then raising the level of calcium results in a more uniform and reproducible culture. Moreover, the change from a low to a high concentration of calcium makes it possible to distinguish two stages of differentiation in the culture: a first stage in which proliferation is the primary event, although differentiation does occur, and a later stage which also involves stratification as well as terminal differentiation.

A cell-culture system is described in which the effects of vitamin A on the differentiation of epidermal cells can be investigated. In this system, rat keratinocytes are triggered to stratify and differentiate by increasing the concentration of calcium in the medium. Excess retinol (RL) or retinoic acid (RAC) in the medium enhances features of the secretory epithelia, whereas a deficiency of vitamin A

accentuates the markers of terminal differentiation of stratified squamous epithelia.

The morphological changes expressed in primary cultures in the presence of excessive and deficient amounts of retinoids and their effects on the terminal differentiation of primary cultures are described.

Methods

Cultures

Keratinocytes were obtained from newborn CFN rats (1–3 days old). The animals were obtained from a colony which has been randomly bred for over 10 years in our laboratory. Skin was removed from the back of the rat and treated overnight with 0.25% crude trypsin (Difco) at 4° C. The epidermis was then separated from the dermis, and the cells were brushed from the dermal side. The cells were then layered on 10% Ficol and centrifuged for 5 min at 16 *g* to separate clumps of basal cells (sediment) from single cells that included differentiated epidermal cells and fibroblasts (supernatant); this procedure was repeated four or five times. The basal-cell fraction was suspended in medium and plated at 5×10^5 cells per 35-mm Corning plastic culture dish [30]. The cultures were grown at 35° C in a humidified 5% CO_2 : air atmosphere. The medium was changed every other day.

Types of media

The cells were cultivated in several media that differed in their concentrations of calcium and retinoids.

Normal medium. Eagle's minimum essential medium (MEM) in Earl's balanced salt solution (EBSS) was supplemented with 10% fetal calf serum (FCS), 10 µg/ml insulin, 10 µg/ml hydrocortisone, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 0.5 µg/ml fungizone. The calcium concentration of this medium was 1.96 m*M*, and the vitamin-A content was 20 ng/ml. (Vitamin A was assayed by Dr. H. Stowe of the Animal Health Diagnostic Laboratory at Michigan State University.)

Low-calcium medium. Calcium-free MEM was supplemented with 10% Chelex-treated FCS [2] and adjusted to 0.09, 0.11, 0.14, 0.17, or 0.20 m*M* by the addition of calcium chloride. Calcium measurements were performed by atomic absorption using the method described in [13] or that of Cali et al. [3]. This medium contained 20 ng/ml vitamin A.

High-retinoid medium. Normal medium was supplemented with 2, 4, 8 or 12 µg/ml trans-RL or trans-RAC. The RL and RAC were solubilized in dimethylsulfoxide (DMSO). The final concentration of DMSO in the medium was 0.02%, and the concentration of calcium in this medium was 1.96 m*M*.

Low-retinoid medium. The FCS added to the MEM was delipidized according to the method of Rothblat et al. [28]. This medium contained 0–3 ng/ml vitamin A, and the concentration of calcium was 1.96 m*M*.

Experimental procedure

Basal cells were plated in low-calcium medium and allowed to grow for 3–5 days to reach 70%–80% confluence. The medium was then replaced by either normal, high-retinoid, or low-retinoid medium. Growth was followed by phase-contrast microscopy and electron microscopy at different time intervals after the replacement of the medium.

Preparations of cultures for electron microscopy. Cultures were fixed in plastic culture dishes with Karnovsky fixative [19] for 1 h at room temperature and then overnight at 4° C. The fixed cultures were rinsed with 0.1 *M* cacodylate (pH 7.4) containing 0.3 *M* sucrose, postfixed with 2% osmium tetroxide, rinsed with buffer, stained with 2% uranyl acetate, rinsed with distilled water, dehydrated in a series of graded ethanols, and embedded in Epon in a culture dish. Thin sections cut in a direction perpendicular to the culture dish were poststained with uranyl acetate and lead citrate. Some cultures were detached from the dish using 5 mg/ml dispase [12] and then fixed, embedded, sectioned, and poststained as already described.

Indirect immunofluorescence

Cultures were grown on glass coverslips (11 × 22 mm), starting with low-calcium medium which was replaced by the other types of medium at confluence as already described. When stratification was observed (2–4 days after the replacement of the medium), the coverslips were rinsed in phosphate-buffered saline (PBS) and fixed for 30 min in buffered 4% formaldehyde at room temperature. The coverslips were then rinsed for 20 min in three changes of PBS, transferred for 5–7 min to acetone at −20° C, and then rinsed again in PBS. The coverslips were incubated for 1 h at room temperature with the monoclonal anti-keratin AE2 ([33]; provided by Dr. T.T. Sun). The coverslips were rinsed in PBS, incubated with fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse IgG (dilution, 1:40), rinsed in PBS, and mounted in glycerine buffered to pH 9.0 with 0.1 *M* Na_2HPO_4.

Results

Phase-contrast observations

Low-calcium medium. Keratinocytes cultivated in 0.11 m*M* calcium formed a typical low-calcium monolayer of distinct cells with large intercellular spaces (the typical cobblestone pattern [16]; Fig. 1a). This culture could be maintained for more than 6 weeks. When the calcium concentration of the medium was lower than 0.11 m*M*, the cells were not viable after a few days. When cultivated in media with 0.14 or 0.17 m*M* calcium, stratified foci were observed after 7–14 days. In medium with 0.20 m*M* calcium, the cultures stratified similarly to those grown at a normal calcium concentration (1.96 m*M*; Fig. 1b, e). We found that 0.11 m*M* calcium was the optimal calcium concentration for maintaining a monolayer of rat keratinocytes.

When the concentration of calcium in the cultures that had been started in low-calcium medium was raised by switching them to normal medium, the intracellular spaces disappeared, the cells became extremely flat, and the cultures started to stratify to form a second layer within 48 h (Fig. 1b, e). Additional layers were formed with time.

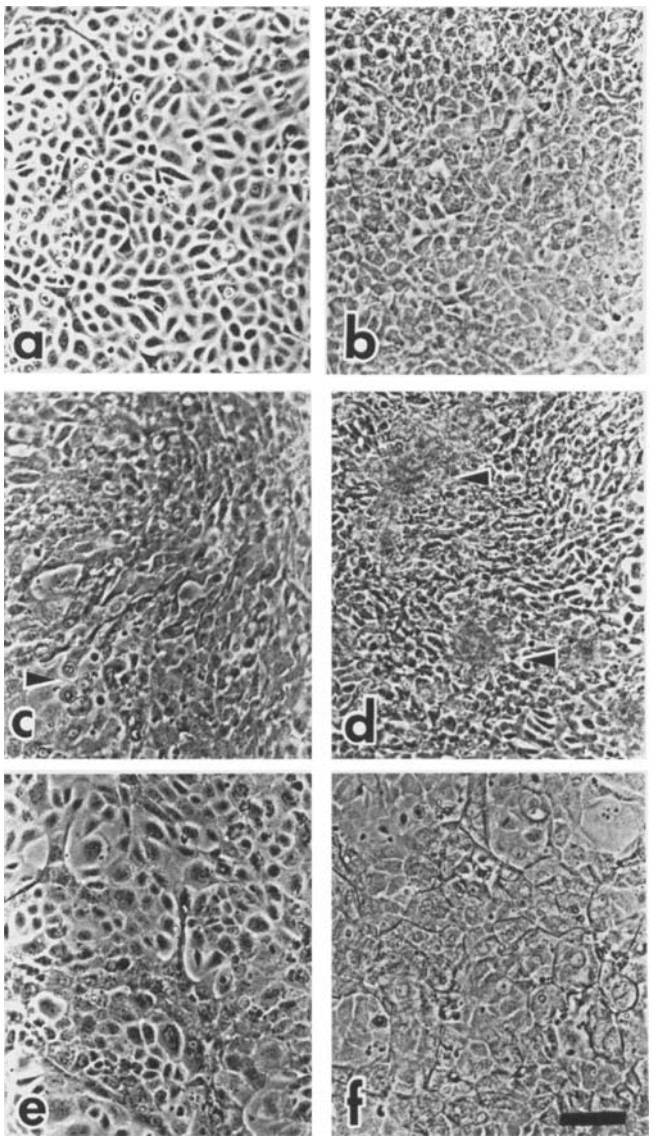

Fig. 1a–f. Rat keratinocytes grown in different types of medium. **a** Five-day-old culture in low calcium medium. Note the small cells and wide intercellular spaces. **b** Culture 3 days after being transferred from low-calcium to normal-calcium medium containing 0.02% DMSO (control for **c** and **d**). This is an area of the culture with no stratification; the cells are flat and large, and the intercellular spaces are narrow. **c** Culture 3 days after being transferred from low-calcium to high-retinoid medium containing 8 μg/ml retinol. Note the elongated and stratifying keratinocytes and sloughing cells (*arrowhead*). **d** As in **c**, but with high-retinoid medium containing 12 μg/ml retinoic acid. The elongated keratinocytes are forming swirl patterns. The sloughing of pieces has started (*arrowheads*). **e** Culture 2 days after being transferred from low-calcium to normal medium (control for **f**). Stratification has started. **f** Culture 2 days after being transferred from low-calcium to low-retinoid medium. Stratification is extensive. Note the top layer of large, angular cells with accentuated cell membranes. *Bar*, 100 μm. ×90

High-retinoid cultures. RAC or RL (at 2, 4, 8, or 12 μg/ml) was added to the medium when the cultures were transferred from low-calcium to normal medium. In these cultures, differentiation and stratification occurred in the presence of high concentrations of retinoids.

When RAC was added to the medium, the cells became elongated and formed swirl patterns with wide intercellular spaces similar to those described for calf-esophagus epithelial cells [23] and fibroblasts [26], but different from those described for mouse keratinocytes [35, 36]; (Fig. 1d). When the concentration of RAC was 12 μg/ml, the culture failed to stratify and did not survive for more than 8–10 days. At lower concentrations, the cultures started to stratify within 48 h. Pieces or scales started to slough from the top of the culture after 10 days in media containing 8 μg/ml RAC or after 2–3 weeks in media containing 4 μg/ml RAC. Single, large, rounded cells sloughed from the cultures continuously, but more so when the concentration of RAC was 8 or 12 μg/ml. In cultures transferred to normal medium, sloughing started after 20 or more days. Similar changes were observed when RL was added to the medium (Fig. 1c); sloughing started 3–5 days later than in RAC-treated cultures. Some of the cultures grown in medium with 4 μg/ml RL did not slough after 3 weeks. Single, large, rounded cells also sloughed from this culture, but to a lesser extent than in the RAC-treated cultures.

Retinoid-treated cultures deteriorated and died earlier than cultures grown in normal medium. At concentrations of 8 or 12 μg/ml and of 4 μg/ml retinoids, the cultures died 15–20 days and 5–7 days earlier respectively than cultures grown in medium without added retinoids (5–6 weeks).

Low-retinoid culture. When the change to normal calcium was coupled with the addition of delipidized serum, stratification started within 48 h, as in cultures grown in other media. The low-retinoid cultures were transparent, i.e., their cells did not contain granules. The cells of the upper layers were large and polygonal, their membranes were accentuated and angular, and the nuclei were small and irregular in shape (Fig. 1f). These cultures survived for only 7–10 days. There was no sloughing of pieces or of single cells. At about 1 week, the cells in those parts of the culture that remained as a monolayer started to deteriorate. One or two days later, the entire culture detached from the dish in one piece; very few cells remained attached to the dish.

Ultrastructure of cultures

Low-calcium culture. The basal cells that formed the monolayer were rounded (Fig. 2a). Cell-to-cell contacts were by very elongated filopodia-like processes. The cell surface facing the medium had long microvilli. The lower membrane of the cells was attached to the plastic substrate by small, bud-like projections (Fig. 3). The part of the membrane that was attached to the plastic was, at times, thickened (possibly hemidesmosomes; see also [4, 17]). No desmosomes were found in the culture. In the lower part of the cells close to the plastic surface, there were many fine filaments (possibly microfilaments). Similar filaments were also seen in some of the elongated microvilli. The nuclei were rounded and often had long, thin indentations. Large, dense bundles of tonofilaments and 'empty' vacuoles surrounded the nuclei. The Golgi zone, consisting of stacks of narrow, smooth membrane cisternae, was found near the nucleus and sometimes inside the indentations. The cells detached from the monolayer were rounded, and their tonofilaments were found exclusively around the nuclei. These cells did not have cornified cell envelopes. In some of these detaching cells, the cytoplasm contained few cell organelles and therefore appeared light.

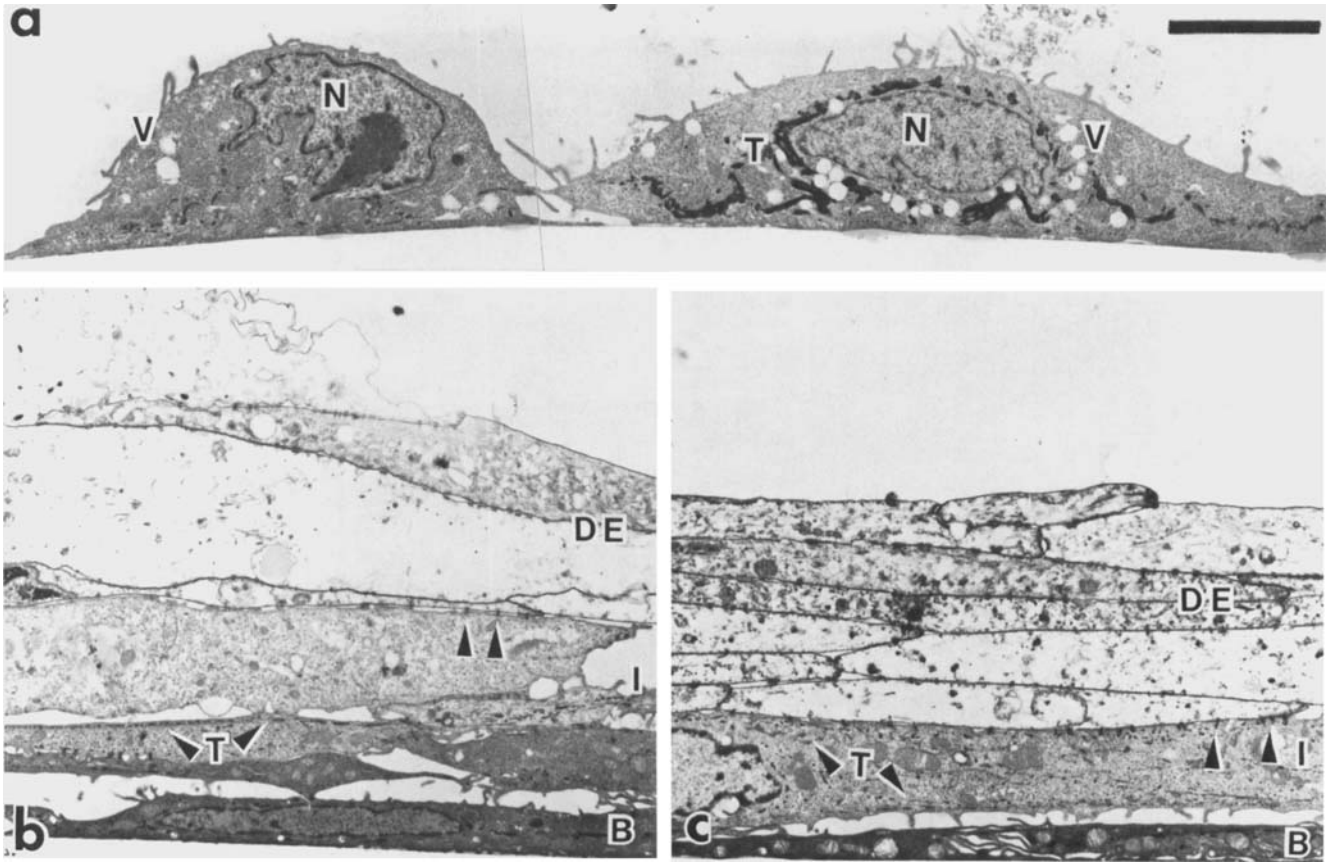

Fig. 2a–c. Electron micrographs of cultures of rat keratinocytes grown in the different types of medium and sectioned perpendicular to the culture dish. **a** Low-calcium culture (11 day old); keratinocytes are rounded and attached to each other by long processes. Tonofilaments (*T*) and empty vacuoles (*V*) surround the nucleus (*N*). **b, c** Keratinocytes were grown in low-calcium medium for 4 days and then transferred to normal (**b**) or low-retinoid (**c**) medium for 7 days. Note the differences in the intercellular spaces, the number of differentiated layers, and the amounts of filaments seen in the two cultures. *B*, basal zone; *I*, intermediate zone; *DE*, differentiated zone; *N*, nucleus; *T*, tonofilaments; the *arrowheads* point to desmosomes. *Bar*, 5 μm. ×4,000

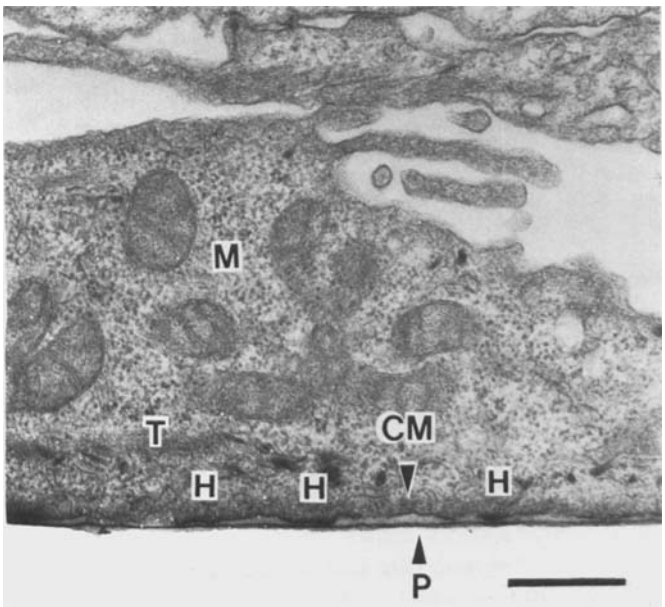

Fig. 3. Micrograph showing the attachment of basal cells to the plastic dish. Part of a basal cell from a culture 3 days after transfer from low-calcium to normal medium showing hemidesmosome-like structures (*H*). Note mitochondria with lucent matrix typical of this growth condition. *P*, plastic dish; *CM*, cell membrane; *T*, tonofilaments; *M*, mitochondria. *Bar*, 1 μm. ×17,000

Normal culture. Several layers of cells were seen 3–4 days after replacing the low-calcium medium (0.11 m*M* calcium) by normal medium (1.96 m*M* calcium). Three distinct zones could be morphologically characterized (Fig. 2b): the basal, intermediate, and differentiated zones [17].

Cells of the basal zone were very flat and were connected to adjacent and upper cells by desmosomes at the ends of microvilli. There were large intercellular spaces. The cells of the basal zone differed from those of the low-calcium monolayer by having desmosomes and containing fewer vacuoles. Their tonofilaments were organized in large dense bundles along the cell membrane (Fig. 4c) and were often connected to the desmosomes. Cells of the intermediate zone were usually flat and had fewer microvilli and less cell organelles. The cytoplasm of these cells appeared lighter than that of the basal cells. Many desmosomes bound these cells both to the basal- and differentiated-cell zones. The cells of the differentiated zone had cornified cell envelopes and were attached to each other by numerous desmosomes. Their cytoplasm contained only very few organelles, and most of them appeared to be at different stages of disintegration. In the uppermost cells, the main components were tonofilaments that, in some areas, were closely associated with the cornified cell envelopes. The tonofilaments of these differentiated cells appeared as groups of fine threads distributed in the cytoplasm (Fig. 5a). Some of these cells contained many such filaments, whereas others had very few

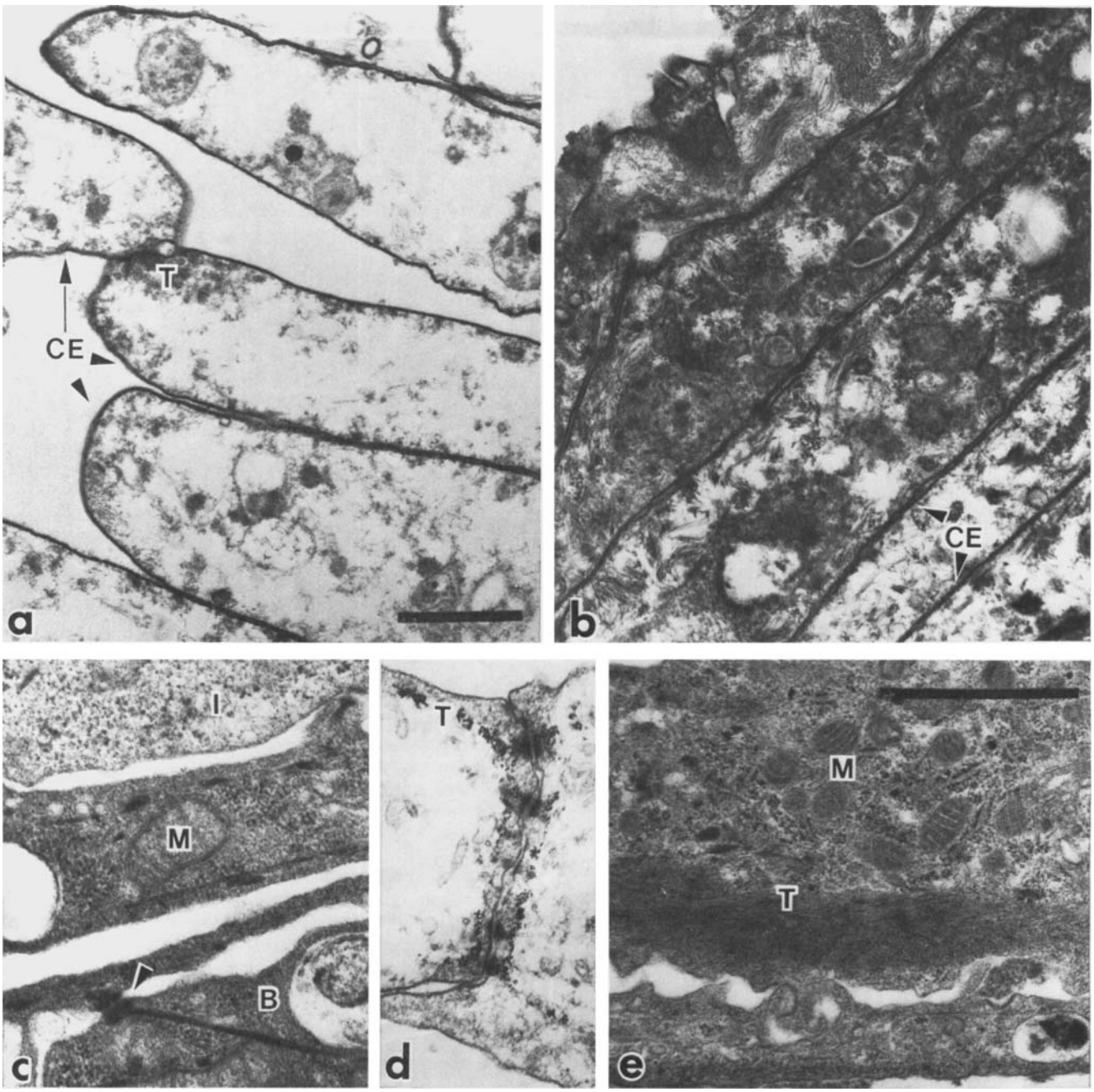

Fig. 4a–e. Details of cultures grown in different types of medium. **a** Cells from the differentiated zone of a culture 27 days after transfer from low-calcium to normal medium. Note the cornified cell envelopes and the small number of tonofilaments. **b** Cells from the differentiated zone of a culture 7 days after transfer from low-calcium to low-retinoid medium. Note the cells packed with tonofilaments. **c** Desmosome (*arrowhead*) connected to tonofilaments in a culture 7 days after transfer from low-calcium to normal medium. **d** Desmosomes between two cells in the same layer of the differentiated zone in a culture 7 days after transfer from low-calcium to low-retinoid medium. **e** Tonofilaments arranged in a wide, diffuse band in a basal cell of a culture 7 days after transfer from low-calcium to high-retinoid medium containing 2 μg/ml retinoic acid. Note mitochondria with dense matrices typical of high-retinoid cultures. *CE*, cornified envelope; *T*, tonofilament; *M*, mitochondria; *B*, basal zone; *I*, intermediate zone. *Bar*, 1 μm. **a**, **b** × 20,000; **c–e** × 33,000

and appeared almost empty. Granules resembling keratohyalin granules were occasionally seen in both the intermediate and differentiated zones.

Retinoid-treated cultures. These cultures started to slough earlier than cultures grown without added retinoids. However, during the first 7–10 days, the number of layers in the retinoid-treated cultures was similar to that in normal cultures. After sloughing had started, most of the cultures were one to four layers thick.

In RL-treated cultures, the basal cells did not differ greatly from those of normal cultures. However, some of the cells had very elongated microvilli and a few narrow desmosomes, and the spaces between cells appeared to be larger than those in normal cultures (Fig. 5a). Several layers of such cells were seen in cultures treated with low concen-

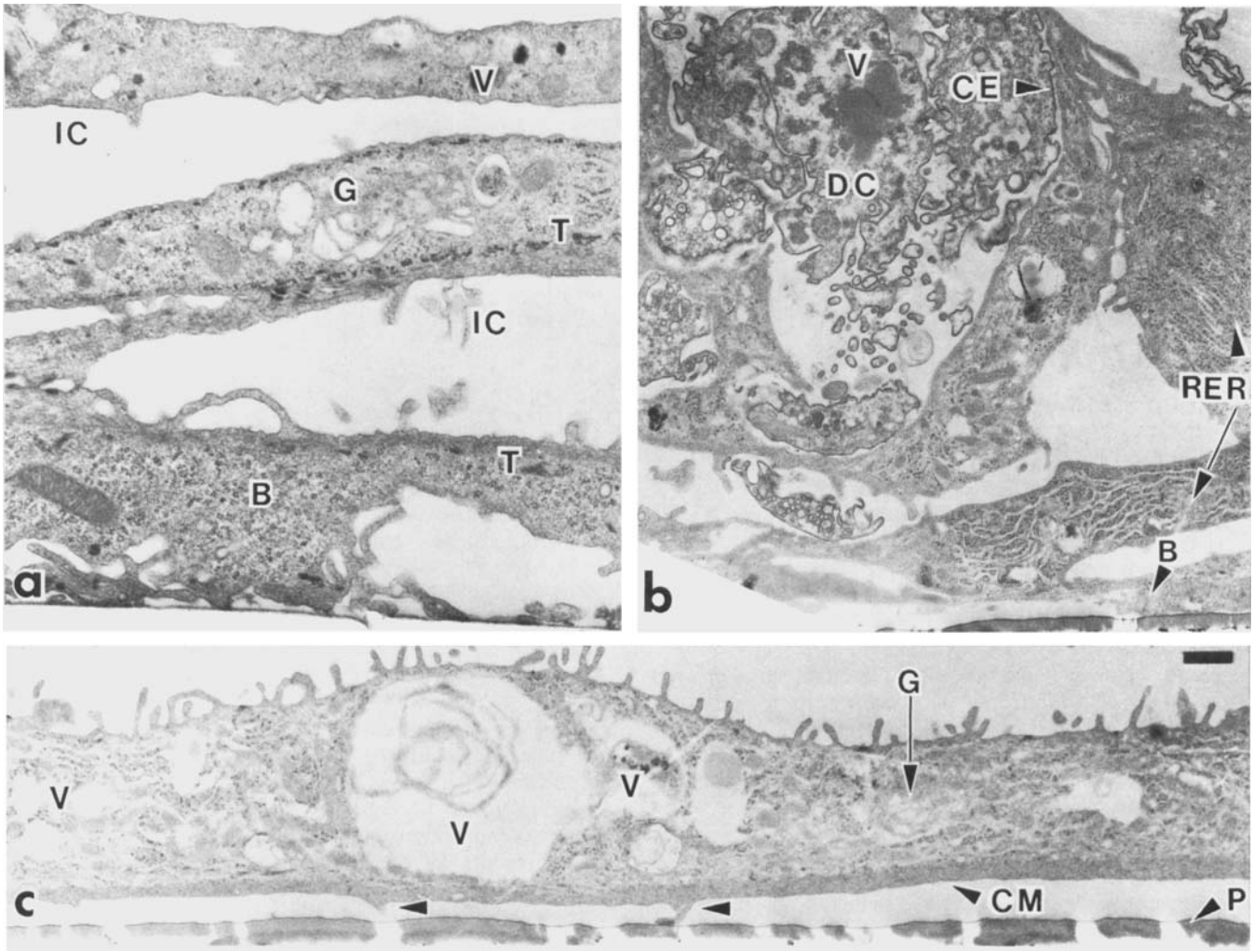

Fig. 5a–c. Micrographs of cultures grown in high-retinoid medium. Keratinocytes were grown in low-calcium medium and then transferred to high-retinoid medium. **a** Culture 2 days after transfer to medium containing 2 μg/ml retinol. Note wide intercellular spaces (*IC*) and dilated Golgi cisternae (*G*). **b** Culture 17 days after transfer to medium containing 4 μg/ml retinoic acid. Note the loss of layered organization, the development of rough endoplasmic reticulum (*RER*), and the disappearance of tonofilaments. Differentiated cells (*DC*) are round, and their membranes are thick (*CE*) and ruffled. **c** Culture 17 days after transfer to medium containing 4 μg/ml retinol. The culture is a monolayer. Note the extensive rough endoplasmic reticulum and the vacuoles (*V*). There are bud-like projections (*arrowheads*) of the basal membrane towards the plastic dish (*P*). *B*, basal zone; *CM*, cell membrane; *T*, tonofilaments. *Bar*, 1 μm, ×7,500

trations of RL, whereas in higher concentrations of RL there were only one to two layers (Fig. 5c). In these cells, the membrane facing the medium had short microvilli. Gap-junctions between cells could be seen at times. In the cytoplasm of the basal cells, there were many membrane-bound ribosomes, the number of which was higher in older cultures grown in 4 and 8 μg/ml RL. Sometimes, cisternae of rough endoplasmic reticulum were formed throughout the cells. More mitochondria were observed in RL-treated cultures than in normal cultures; many of these mitochondria had a very dense matrix. There was also an increase in the number of vacuoles and secondary lysosomes.

In the RL-treated cultures, no intermediate zone was found, and all of the differentiated cells appeared to be in various stages of detachment from the culture, either as flattened scales made from one to three differentiated layers or as single rounded cells with thickened cell membranes. These scales and cells tended to separate from the culture while the tissue was being processed for electron microscopy. The detached differentiated cells had thick cell membranes (possibly cornified cell envelopes) and sometimes contained thick aggregates of short tonofilaments. Other detaching cells either appeared to be empty or contained granules.

Cultures exposed to RAC retained one to two cell layers after sloughing. At an RAC concentration of 8 μg/ml, there was a loss of the layered organization, and some differentiated cells were observed to be surrounded by undifferentiated cells (Fig. 5b). These undifferentiated cells differed from those of normal cultures. The cells had an extensive rough endoplasmic reticulum, an extensive Golgi zone with dilated cisternae (Fig. 6b), many mitochondria with dense, dark matrices, and vacuoles. Few tonofilaments were observed in cultures treated with RAC, and a large proportion of those found did not form the typical bundles observed in the cultures (Fig. 4e). The filaments found in the RAC-treated cultures formed diffuse wide bands. In 17-day-old cultures grown in medium containing 4 or 8 μg/ml RAC,

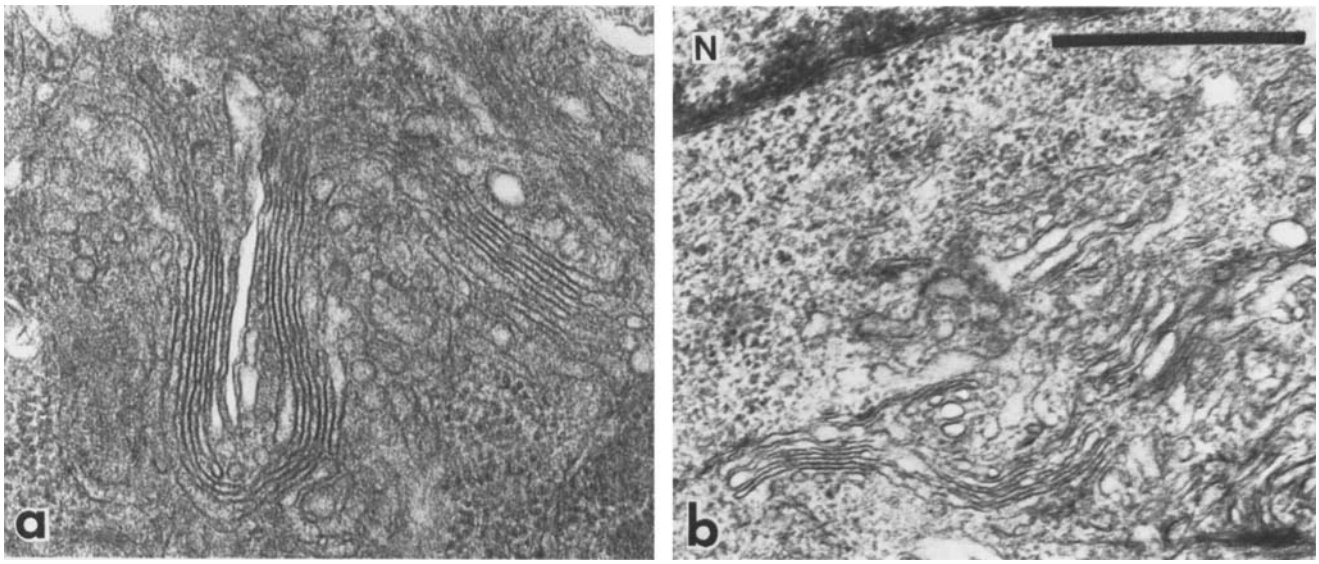

Fig. 6a, b. Golgi area of keratinocytes in culture. **a** Part of a basal cell from a culture 2 days after transfer from low-calcium to normal medium and then detached with dispase. Note the densely packed narrow cisternae. **b** Part of a cell from the basal zone of a culture 2 days after transfer from low-calcium to high-retinoid medium containing 2 μg/ml retinol and processed as in **a**. Note the loosely packed and dilated cisternae. *N*, nucleus. *Bar*, 1 μm. × 33,000

no filaments of any kinds were seen. The differentiated cells (attached or detaching) had thick cell membranes that resembled cornified cell envelopes, but the cells were round and the membranes were ruffled. These cells contained many vacuoles of different sizes and shapes, and sometimes exhibited short, thick tonofilaments.

Low-retinoid cultures. Cultures grown in medium containing delipidized serum stratified very quickly. The number of differentiated layers was higher than that in cultures of the same age that were grown in normal medium (Fig. 2c). The intercellular spaces were narrower than those in cultures grown in other media. The morphology of the cells of both the basal and intermediate zone was similar to that of the normal culture. The differentiated layers consisted of very flat cells with cornified cell envelopes. These cells were sometimes packed with tonofilaments (Fig. 4b) and were connected to each other by many desmosomes (Fig. 4d). In 3-day-old cultures, there was one layer of basal cells and one to three layers in the intermediate zone. In 7-day-old cultures, the cells attached to the plastic substratum resembled cells of the intermediate zone. After 7–10 days, most of the undifferentiated cells disappeared due to either differentiation or death. The differentiated layers appeared as one piece floating in the medium.

Indirect immunofluorescence. Cultures from low-calcium medium and cultures 2–3 days after the switch to each of the other media were stained with the monoclonal anti-keratin AE2. The large polygonal cells in the uppermost layer of the cultures grown in low-retinoid medium reacted with the anti-keratin AE2 (Fig. 7). In many cells, the staining appeared as large round granules; in other cells, it appeared as numerous small granules or was diffused over all of the cytoplasm, leaving the nucleus area unstained. In some cases, small aggregates were seen along the membranes between adjacent cells whose cytoplasm had not been stained by the antibody. Cultures grown either in normal medium or in the presence of high retinoid concentrations did not react with the anti-keratin AE2.

Discussion

Several reports have described the effect of raising the concentration of calcium on the differentiation of keratinocytes in culture [15, 16, 36]. However, these reports do not describe in detail the organization and ultrastructure of the cultures which were obtained after the change in the calcium concentration. In the culture system presented here, the stratification and terminal differentiation of rat keratinocytes is triggered by raising the calcium concentration after a confluent monolayer has been established in the low-calcium medium. The rise in calcium concentration, when coupled with the addition of retinoids or the omission of vitamin A from the medium, influences the initiation of stratification and terminal differentiation.

In this study it was shown that rat keratinocytes transferred from low-calcium to normal-calcium medium produced thriving cultures whose tissue organization and ultrastructural characteristics did not differ from those reported for either mouse or human epidermal cells grown in normal-calcium medium (for a review, see [17]). After the change in the calcium concentration, the cultures stratified and further differentiated to form the three zones (basal, intermediate, and differentiated) typical of differentiating keratinocytes in culture. The cells had tonofilaments, desmosomes, and cornified cell envelopes. The advantages of using a protocol involving a change in calcium concentration are that a more uniform and reproducible culture is obtained and that the initial steps of cultivation, which include plating, attachment, proliferation, colony formation, and confluence, can be separated from the later phase, which includes stratification and terminal differentiation. Moreover, the cells in the monolayer are uniformly exposed to the medium and are more accessible to any changes in its composition.

The cell-culture system presented here developed most of the morphological features (Table 1) that are induced by an excess of vitamin A in epidermal cells. In the presence of an excess of retinoids (2–8 μg/ml), the cultures stratified

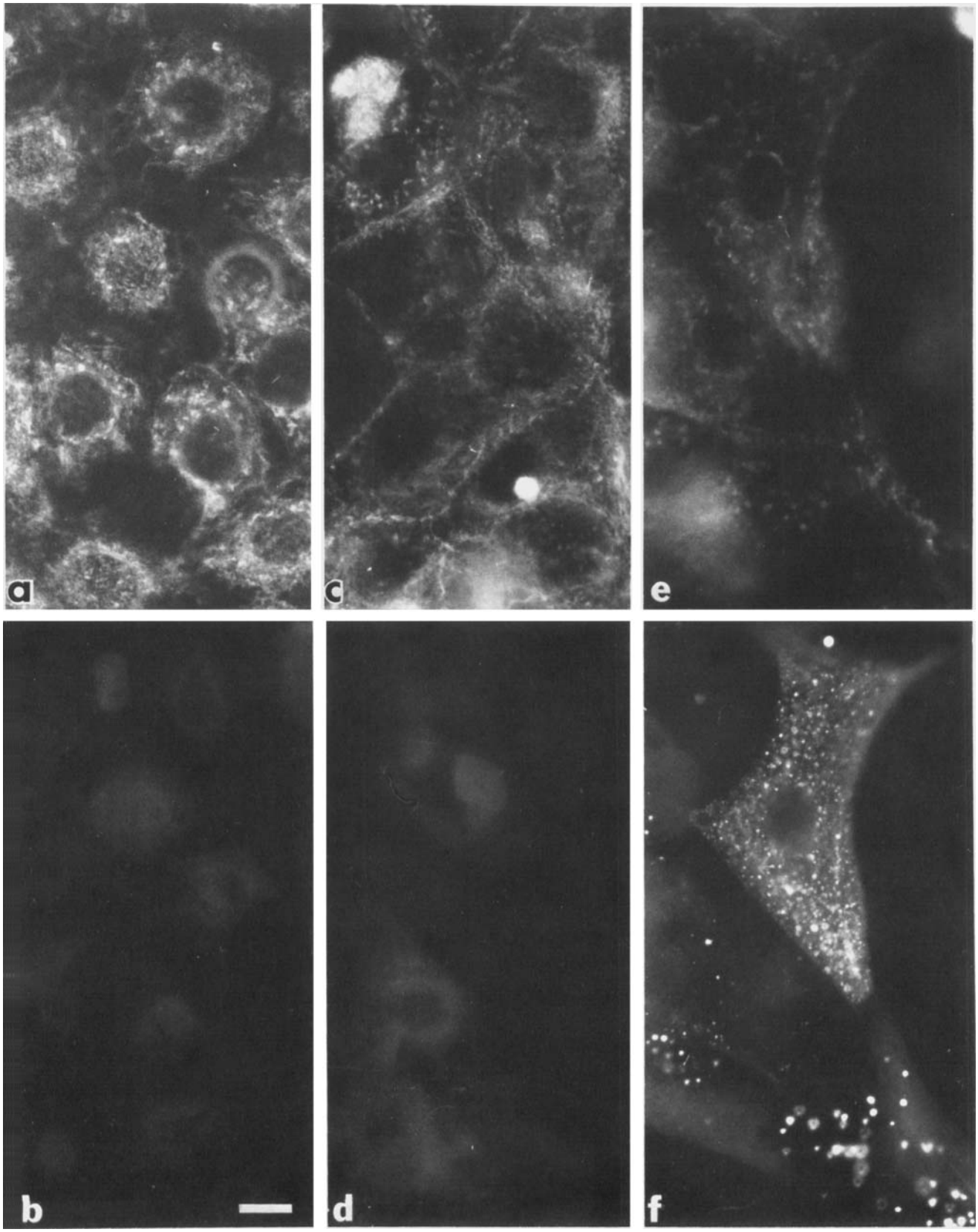

Fig. 7a–f. Indirect immunofluorescence of AE2 anti-keratin in keratinocytes grown in different types of medium. **a**, **b** Keratinocytes grown in low-calcium medium for 6 days (**a** no FITC filter; **b** with FITC filter). **c**, **d** Keratinocytes 2 days after transfer from low-calcium to normal medium. (**c** no FITC filter; **d** with FITC filter). **e**, **f** Keratinocytes 2 days after transfer from low-calcium to low-retinoid medium. (**e** no FITC filter; **f** with FITC filter). *Bar*, 10 μg. × 880

Table 1. Summary of characteristics of the cultures grown in the different types of medium

Low-calcium culture (0.11 m*M*, 3–5 days)	Normal culture (20 ng/ml vitamin A)[a]	High-retinoid culture (2–12 μg/ml RL or RAC)[a]	Low-retinoid culture (1–3 ng/ml vitamin A with delipidized serum)[a]
Number of layers[b]			
Monolayer	1–2 B 1–3 I 3–10 DE	1–4 B	1 B 1–3 I 3–10 DE
Sloughing			
Single cells at all times	Squames after 20 days	Single cells[c] and squames after 5–10 days	No sloughing
Desmosomes along the cell membrane in section			
None	3–5 B 5–15 I 10 or more DE	0–5 narrow desmosomes	5–10 B 10–20 I 20 or more DE
Tonofilaments in basal cells			
Bundles around the nucleus	Bundles along cell membrane	Very few short, thick bundles or diffused wide bands[d]	Bundles along cell membrane
Tonofilaments in differentiated cells			
——	Scattered bundles in all cytoplasm	Occasionally	Very high density in all cytoplasm
Ribosomes			
Free + few membrane bound	Free + few membrane bound	Frequently extensive endoplasmic reticulum	Free + few membrane bound
Golgi areas			
Narrow cisternae	Narrow cisternae	Large area of cell, wide cisternae	Narrow cisternae
Vacuoles			
Around nucleus	Occasionally in basal and intermediate layers	Many all over cytoplasm, sometimes with low-density material	Very few
Staining with AE2			
Negative	Negative	Negative	Staining of cells of upper layers

B, basal zone; I, intermediate zone; DE, differentiated zone; RL, retinol; RAC, retinoic acid

[a] Calcium concentration, 1.96 m*M*

[b] The number of layers increased with the age of culture

[c] The sloughing started earlier in the higher retinoid concentrations

[d] In older cultures, some cells lost their tonofilaments

at similar rates as those of control cultures but had fewer and smaller desmosomes, wider intercellular spaces, and exhibited earlier desquamation. Yuspa et al. [36] have reported the initial suppression of stratification and desquamation in mouse keratinocytes treated with retinyl acetate in low-calcium medium and then induced to stratify by raising the calcium concentration. However, the early desquamation and wide intercellular spaces described in the present study have also been reported in conventional cultures of human keratinocytes treated with RAC [20, 23].

The decrease in the amount of tonofilaments and the formation of wide and loosely packed bundles of tonofilaments seen in the retinoid-treated cultures of the present study have also been reported in retinoid-treated skin explants [1]. Retinoids induce a decrease in the ratio of prekeratin and keratin to the total solubilizable proteins in treated cultures [21]. Retinoids also inhibit the synthesis of high-molecular-weight keratins, promote the synthesis of low-molecular-weight keratins in culture [10], and inhibit the cross-linking of keratins [34]. It is therefore possible that these changes are expressed in the morphology of tonofilaments as well as in their abundance. The high density of tonofilaments observed in the differentiated cells found in the vitamin-A-deficient cultures of the present study supports this conclusion.

Retinoids reduce the formation of cornified cell envelopes [11, 34]. In the present study, thick cell membranes that appeared to be cornified cell envelopes were seen in the cells that detached from the retinoid-treated cultures. These cells did not have other features characteristic of differentiated cells; they were not flat, they did not necessarily contain tonofilaments or have desmosomes, and they often contained numerous vacuoles. These vacuoles were PAS and oil-red positive (unpublished result).

As the features of terminally differentiated keratinocytes disappeared in the retinoid-treated cultures, there was an enhancement of secretory-cell features (see also [35]). In the retinoid-treated cultures, many cells had membrane-bound ribosomes that were often arranged as a rough endoplasmic reticulum: the Golgi area was large and had very dilated and loosely packed cisternae, while the cisternae in untreated or low-calcium cultures were narrow and densely packed. The large number of mitochondria with dense matrices and the secondary lysosomes indicate a high metabolic activity associated with the changes that these cells had endured.

Keratinocytes that stratified and differentiated in the absence of vitamin A (medium supplemented with delipidized serum) had more features characteristic of fully differentiated stratified squamous epithelium than those grown in normal medium. More differentiated layers of closely associated flat cells were formed. Numerous desmosomes and tonofilaments were observed in the intermediate and differentiated zones. The uppermost cells of these cultures reacted with the monoclonal anti-keratin AE2, which indicates that they contained keratins that are markers of terminal differentiation [9, 33]. In vivo, a vitamin-A deficiency causes keratinization of epithelial tissues that do not keratinize normally and hyperkeratinization of tissues that are normally keratinized [5, 32]. Furthermore, keratinocytes in culture grown in a medium supplemented with delipidized serum exhibit enhanced terminal differentiation [10]. However, it has been shown that fatty acids added to medium supplemented with delipidized serum inhibit morphological

differentiation of neuroblastoma cells in culture [24]. It is possible, therefore, that other lipids that are removed from the serum by the delipidization process may have contributed to the higher degree of differentiation achieved.

Cultures of human keratinocytes grown in normal medium are stained by the monoclonal antibodies AE1 and AE3, but not by AE2 [33], probably because these cultures do not synthesize the 65- to 67-kilodalton (K) keratins [10]. The pattern of staining observed in human keratinocytes in culture reveals the cytoskeletal net formed by the keratin filaments [29, 33]. In the low-retinoid culture described in the present study, the staining pattern of AE2 was granular or diffuse (Fig. 7). This may indicate that the keratin fibers in the terminally differentiated cells of the low-retinoid cultures were organized differently from those in the undifferentiated cells (Fig. 4). In the undifferentiated cells, the tonofilaments were found mainly along the cell membrane, whereas the tonofilaments of the differentiated cells were seen throughout the cytoplasm. The latter may have been the reason for the granular pattern observed when the low-retinoid cultures were stained by AE2. Dale and Sun [7] have reported that the anti-keratin, AE2, cross-reacts with filaggrin. It is possible that keratohyalin contributed to the staining of the differentiated cells by AE2. However, very few keratohyalin granules were observed in the cultures under all treatment conditions, whereas only cells of the low-retinoid culture were stained by the AE2 anti-keratin. It is therefore more likely that the staining pattern was a result of the presence of the 56.5-K and 65- to 67-K keratins and not of filaggrin.

This system responds to changes in vitamin-A concentrations in a manner similar to intact skin and should be suitable for the study of the site(s), the time, and the mechanism by which retinoids affect epidermal differentiation.

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