

STRATIFICATION, SPECIALIZATION, AND PROLIFERATION OF PRIMARY KERATINOCYTE CULTURES

Evidence of a Functioning In Vitro Epidermal Cell System

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ABSTRACT

A population of neonatal mouse keratinocytes (epidermal basal cells) was obtained by gentle, short-term trypsin separation of the epidermal and dermal skin compartments and discontinuous Ficoll gradient purification of the resulting epidermal cells. Over 4–6 wk of culture growth at 32°–33°C, the primary cultures formed a complete monolayer that exhibited entire culture stratification and upper cell layer shedding. Transmission and scanning electron microscopy demonstrated that the keratinocyte cultures progressed from one to two cell layers through a series of stratification and specialization phenomena to a six to eight cell layer culture containing structures characteristic of epidermal cells and resembling in vivo epidermal development. The temporal development of primary epidermal cell culture specialization was confirmed by use of two histological techniques which differentially stain the specializing upper cell layers of neonatal mouse skin. No detectable dermal fibroblast co-cultivation was demonstrated by use of the leucine aminopeptidase histochemical technique and routine electron microscope surveillance of the cultures. Incorporation of [³H]thymidine ([³H]Tdr) was >85% into DNA and was inhibited by both 20 μM cytosine arabinoside (Ara-C) and low temperature. Autoradiography and 90% inhibition of [³H]Tdr incorporation by 2 mM hydroxyurea indicated that keratinocyte culture DNA synthesis was scheduled (not a repair phenomenon). The primary keratinocytes showed an oscillating pattern of [³H]Tdr incorporation into DNA over the initial 23–25 days of growth. Autoradiography demonstrated that the cultures contained 10–30% proliferative stem cells from days 2–25 of culture. The reproducibility of both the proliferation and specialization patterns of the described primary epidermal cell culture system indicates that these cultures are a useful tool for investigations of functioning epidermal cell homeostatic control mechanisms.

KEY WORDS primary epidermal cell system ·
proliferation · specialization ·
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The skin consists of two adjacent tissue compartments: the uppermost epidermis and the supportive dermis. The epidermal layer has a population

of stem cells (basal) whose progeny undergo specialized alterations as they form the upper stratified areas of the tissues (spinous, granular, and corneal layers). This proliferation-specialization pattern makes the epidermis an excellent model tissue for investigations of the basic control mechanisms of normal and abnormal cell proliferation, sequential differentiation (specialization), and the role of a variety of intracellular and extracellular modulators on these functions.

To study epidermal homeostatic control mechanisms, the epidermal cells (keratinocytes) must be isolated from the influence of the underlying dermal environment so that isolated epidermal cell functions, i.e., proliferation and production of proteins unique to the specialized epidermis (i.e., the keratins), can be investigated and manipulated.

Numerous mammalian cutaneous tissues and *in vivo* techniques have been explored: explant cultures (11, 12, 14, 21), short-term cultures of trypsinized keratinocytes (6, 19, 23), and cultures of dedifferentiated keratinocytes (13, 34, 35). Many of the reported keratinocyte cultures contain co-cultivated fibroblasts (22, 32, 44) and require the use of vitamin A acid (4) or EDTA and trypsin treatments (25) to extend keratinocyte proliferative capacity. Systems defining exogenous cell influences on keratinocyte functions have been reported by Rheinwald and Green (37, 38) and Sun and Green (41) using 3T3 cell irradiated feeder layers and epidermal growth factor, and by Karasek and Charlton (23) and Melbye and Karasek (31) who studied keratinocytes cultivated with fibroblasts or fibroblast culture-enriched medium.

We required for our investigations of epidermal cell physiology an isolated and functioning epidermal cell system. It is the purpose of this report to describe the preparation and general proliferative and specialized characteristics of the primary epidermal cell system that we have developed for the purpose of our studies of keratinocyte function.

MATERIALS AND METHODS

Reagents

Neonatal mice were obtained from the Dermatology Department (University of Michigan Medical School) BALB/c colony. Medium 199 (M-199, modified, Earle's salt, glutamine), fetal calf serum (FCS), penicillin and streptomycin (P & S), phosphate-buffered saline (PBS) (Dulbecco's), and Lux plastic cover slips were from Flow Laboratories Inc. (Rockville, Md). Earle's balanced salt

solution (BSS) and 0.4% trypan blue were from Grand Island Biological Co. (Grand Island, N. Y.). Trypsin 1:250 was obtained from Difco Laboratories (Detroit, Mich.). Collagenase, DNase, Ficoll 400, fast blue B (*O*-Dianisidine, tetrazotized), *L*-leucyl-4-methoxy- β -naphthylamide HCl, cytosine arabinoside (Ara-C), hydroxyurea, and Dowex 1 \times 2 were purchased from Sigma Chemical Co. (St. Louis, Mo.). Orange G, phosphotungstic acid, aniline blue, acid fuchsin, and all photographic chemicals were obtained from Eastman Organic Chemical Div., Eastman Kodak Co. (Rochester, N. Y.). Erythrosin B, iodine, and other histological stains were from Matheson Gas Products (East Rutherford, N. J.). Saffron (Chroma-Gesellschaft) is distributed by Roboz Surgical Instrument Co., Inc. (Washington, D. C.). Uranyl acetate was obtained from Mallinckrodt Inc. (St. Louis, Mo.) and Epon 812 from Ladd Research Industries (Burlington, Vt.). [3 H]thymidine ([3 H]Tdr) (5-10 and 60 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.). Corning plastic products (Corning Glass Works, Corning, N. Y.) were used in all phases of cell isolation and growth. All other chemicals were reagent grade.

Isolation of Cells

EPIDERMAL CELL CULTURES: 40-60 Neonatal mice (0-24 h old) were washed twice with pHisoHex (Winthrop Laboratories, N. Y.) and 70% ethanol, and were anesthetized by submersion in ice. The whole skin was removed under sterile conditions, cut into several pieces, and floated dermis down on 5 ml of 0.25% trypsin in M-199 equilibrated for air PCO_2 (partial pressure of CO_2). An additional 5 ml of cold trypsin solution was added before incubation. After 1-h incubation at 37°C (humidified), the trypsin was aspirated and 5 ml of M-199 plus 13% FCS was added. The dermis was manually separated from the epidermis, and the keratinocytes were removed by gentle shaking and scraping of the epidermis. Cell suspensions from the dishes were pooled in 50-ml conical tubes, centrifuged, and each pellet was re-suspended in 10 ml of M-199 + FCS. The suspension, representing 15 mice, was layered on top of a discontinuous Ficoll gradient (4°C). The gradient consists of 5 ml of 20% and 10 ml of 18, 15, and 12% Ficoll, bottom to top, in a 50-ml conical tube. A stock solution of sterile 30% Ficoll (g/vol) in Dulbecco's PBS was diluted with M-199 + 13% FCS to give the appropriate densities. Gradients were spun at 4°C at 258 *g* for 30 min. Cellular debris and contaminating fibroblasts were found at the 12-15-18% Ficoll gradient interfaces. The keratinocytes at the bottom of the tube (20% Ficoll) were pooled from the gradients, washed with 50 ml M-199 + 13% FCS, and counted using 0.06% trypan blue in medium. The cell pellet was suspended to give 5×10^6 trypan blue-excluding cells/ml. 1 ml of cell suspension was inoculated into a T-25 flask containing 4 ml of M-199 + 13% FCS + 50 IU/ml of penicillin and 50 μ g/ml of streptomycin. Flasks were gassed with 5% PCO_2 in air and closed. The

cells were grown at 33°C and the medium was changed every 2nd day. 40–50 T-25 flasks result from one culture preparation.

DERMAL FIBROBLAST CULTURES: The trypsin-separated dermis was minced and suspended in 0.25% collagenase in Dulbecco's PBS. The suspension was agitated in a 125-ml Erlenmeyer flask for 30 min at 37°C, at which time 1×10^6 dornase units of DNase was added to the suspension and the incubation was continued (5 min). The suspension was filtered through gauze and centrifuged at 258 g for 5 min. The pellet was resuspended in 10 ml of M-199 + 13% FCS and layered over a Ficoll gradient (as above). Cells from the 12–15% Ficoll interface were plated as fibroblasts (1×10^6 cells/T-25 flask; M-199 + 13% FCS and P & S, 5% PCO₂ in air gassing). Cultures were fed every 2nd day.

Methods of Assay

To measure the total DNA and protein content of the cultures, the medium was aspirated and the cell layers were rinsed three times with cold Dulbecco's PBS. The cells were scraped into 6 ml of 6% TCA, centrifuged, and the DNA in the pellet was resuspended and hydrolyzed (3% PCA [perchloroacetic acid], 95°C for 15 min). After centrifugation, the supernate was assayed (two dilutions) for DNA by the method of Burton (3). The pellet was dissolved in 1 N NaOH and was assayed in triplicate for total protein by the method of Lowry et al. (26).

To determine the incorporation of [³H]Tdr into keratinocyte culture DNA, the monolayers were pulsed for the specified time with 1 μCi/ml of either 5–10 Ci or 60 Ci/mmol [³H]Tdr. The cells were scraped off the flasks with 4 ml of cold 6% TCA. The precipitate was washed twice with 2 ml of 6% TCA. 1 ml of the 6% TCA fraction was counted to determine the [³H]Tdr acid-soluble fraction. The pellet was hydrolyzed with 3% PCA, and aliquots of the supernate were counted to determine the incorporation of label into DNA and were assayed colorimetrically for DNA.

To ascertain whether any label was incorporated into RNA, a modification of the method of Schmidt and Thannhauser (40) was used. The 6% TCA pellet obtained from [³H]Tdr pulsed-labeled cells was dissolved in 1.0 ml of 0.2 N NaOH and hydrolyzed at 100°C for 20 min. After cooling, 400 μl of 30% TCA was added. The supernate was counted for determination of label present in RNA, and the pellet was processed for DNA and protein analysis, and for quantitation of label incorporated into DNA.

The method of fractionation of deoxythymidine nucleotides by column chromatography was that of Duell et al. (8). After ether extraction, 4–5 ml of the acid-soluble fraction of [³H]Tdr-pulsed cultures was chromatographed on Dowex 1 × 2 (200 mesh) Cl form columns (0.6 cm inside diameter × 6 cm length). Radioactive label and added cold tracer deoxythymidine nucleotides were sequentially eluted with four 3-ml fractions of

water (Tdr); 0.01 N HCl, pH 2.0; 0.05 N HCl, pH 1.31 (deoxythymidine monophosphate [dTMP]); 0.1 N HCl, pH 1.09 (dTMP); 0.2 N HCl, pH 0.54 (deoxythymidine diphosphate [dTDP]) and 0.5 N HCl, pH 0.20 (deoxythymidine triphosphate [dTTP]). 90% of the added tracer deoxythymidine nucleotides and 90–100% of the applied radioactivity was recovered from the columns.

All fractions were counted using dioxane-naphthalene-based scintillation fluid in a Packard Tri-carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) operating at 40% efficiency for tritium. Counts were corrected for acid quench by use of an automatic standard and quench standards.

Histological and Histochemical Stains

The Kreyberg stain was done as reported by Kreyberg (24). The flasks were washed twice with Dulbecco's PBS and allowed to drain. After fixation with acetic alcohol (50 ml of glacial acetic acid plus 150 ml of 80% ethanol) for 1 h, cell layers were rinsed once with 70% ethanol followed by distilled water (2 ×). The flasks were flooded with Mayer's hematoxylin for 4 min; the stain was aspirated and the cultures were rinsed two to three times with tap water and checked for nuclear staining. The cells were overlaid with 1.0% aqueous erythrosin (3 min) and, after removal of the stain, rinsed, in series, with tap water, 80% ethanol, and tap water. The cell layers were flooded with saffron solution (12 g of saffron/600 ml water boiled for 2 h, cooled and filtered, plus 6 ml of 5.0% tannic acid and 6 ml of 4.0% formaldehyde). After 3 min, the flasks were drained and rinsed with absolute ethanol (3 ×). Cryostated neonatal mouse skin was treated in an identical manner.

The acid fuchsin-aniline blue-orange G stain was that of Ayoub and Shklar (2). Washed monolayers and cryostated neonatal mouse skin were fixed from 2 to 16 h in Zenker's solution. The material was washed with tap water for 14 hours, exposed to 0.5% alcoholic iodine solution for 15 min and 5.0% thiosulfate solution for 3 min. After a 10-min rinse in tap water, monolayers and tissue sections were stained in 5% acid fuchsin for 20 min. The material was then directly exposed to a 0.5% aniline blue, 2% orange G, 1% phosphotungstic acid solution for 40 min. Monolayers and sections were washed three times with 95% ethanol and twice with absolute ethanol.

The leucine aminopeptidase stain was done as described by Jacquemont and Pruniéras (20). The cultures were washed twice with Earle's BSS and incubated for 1 h at 37°C in a solution (final pH = 7.0) containing 1.25 ml of substrate (8 mg/ml of L-leucyl-4-methoxy-β-naphthylamide-HCl in deionized water); 12.5 ml of 0.1 M PO₄ buffer, pH 6.5; 10 ml of 0.9% NaCl; 1.25 ml of 2×10^{-2} M KCN; 12.5 mg of fast blue B). The substrate was omitted as a control. After incubation, the solution was decanted and the flasks were rinsed with Earle's BSS, flooded with 0.2 M CuSO₄ solution for 2 min, and rinsed with Earle's BSS. The cultures were fixed with

2% osmium tetroxide vapors for 2 min, and rinsed with deionized water and absolute ethanol.

The various steps of the dissociation procedure was monitored by hematoxylin and eosin staining of whole skin, isolated epidermis, and resulting epidermal cell fractions.

Transmission Electron Microscopy

Cultures were flooded with a fresh solution of 4.0% glutaraldehyde and 1.0% osmium tetroxide in 0.1 M sodium cacodylate buffer at pH 7.2 (4°C) for 1 h. Prestaining was done with 0.5% uranyl acetate in 0.04 M veronal acetate buffer at pH 6.1 for 30 min. Dehydration was done at 4°C and 2-min periods of, respectively, 25, 70, 95, and 100% (2 ×) ethanol. After infiltration (vol/vol absolute ethanol to Epon 812 mixture), the cultures were embedded in Epon 812 mixture (5 min, 3 ×) (27) and polymerized for 48 h at 60°C. Areas were reoriented perpendicular to the cell layer surface in Epon blocks with Epon mixture. After hardening at 60°C overnight, thin sections were cut on LKB ultratome III (LKB Instruments, Inc., Rockville, Md.), with a diamond knife, and picked up on Formvar-coated grids. All sections were stained with uranyl acetate and lead citrate (36). Sections were examined with a Philips 300 electron microscope at 60 kV.

Scanning Electron Microscopy

Primary epidermal cells were grown in T-25 flasks containing two or three plastic cover slips. The flasks were flooded with fresh growth medium and an equal volume of 6% glutaraldehyde containing 0.05 M PO₄ buffer (pH 7.2). After 30–45 min of fixation at room temperature, the cover slips were transferred to glass petri dishes where they were briefly washed in 0.2 M PO₄ buffer (pH 7.2). The cells were dehydrated by flooding with increasing concentrations of ethanol (5, 10, 20, 40, 60, 80, 100% [2 ×]), 5 min at each concentration. Amyl acetate infiltration was done by a series of 2-min washes in, consecutively, 20% amyl acetate/80% ethanol; 50% amyl acetate/50% ethanol; 80% amyl acetate/20% ethanol; 100% amyl acetate (2 ×). The amyl acetate was replaced by liquid CO₂ (1), with only small fragments of the plastic cover slips being carried through this part of the process. After mounting on an SEM stud, the cell layer was coated with a gold-palladium alloy (3 min). Scanning electron microscopy was done on a JSM-U5 SEM, working at 25 kV and 45° tilt.

Autoradiography

Flasks containing three to four Lux plastic cover slips were pulsed with 1 μCi/ml [³H]Tdr (sp act 60 Ci/mmol) for 15–20 h. The cover slips were removed, washed twice with Dulbecco's PBS, and fixed for 30 min in acetic alcohol. After two rinses in 80% ethanol, the cover slips were fixed in 6% TCA (30 min), rinsed three

times with distilled water, and air dried.

For autoradiography, the method of Durie and Salmon (9) was employed. The cover slips were stained with hematoxylin and eosin.

RESULTS

Isolation of Cells

Trypsinization cleaves full thickness neonatal mouse skin at the epidermal-dermal junction. Gentle shaking and scraping of the isolated epidermis removes the basal and spinous cell layers while the granular layer and stratum corneum remain intact. Staining of the cell pellet obtained after Ficoll gradient centrifugation showed that the inoculum consists mostly of round cells containing a large nucleus (basal cells). A minimum number of flat polygonal cells with a prominent nucleus (spinous) also layers with the 20% Ficoll fraction. 80% of the basal cells exclude trypan blue while a majority of the spinous cells take up the dye.

Variations in the trypsinization time (i.e., 40–90 min) qualitatively affected the cell suspension. 40-min trypsinization resulted in decreased digestion of the epidermal-dermal junction and yielded a suspension consisting of clumps of 20–40 keratinocytes, rendering cell enumeration and inoculation less precise. A digestion time of 90 min or more resulted in almost complete dermal-epidermal shearing so that the epidermis often floated off the dermis, and the basal cells were lost upon subsequent trypsin solution aspiration. The cell suspension consisted of single cells which, upon Ficoll gradient purification, give a broader zone of cell dispersion, i.e., one-half of the 18% and all of the 20% Ficoll gradient, such that less sharp cell purification resulted. 60-min trypsinization yields suspensions consisting of two to four cell clumps and single cells, all of which are easily countable, give sharp Ficoll gradient separation and show the largest percent of trypan blue exclusion. Therefore, 1-h trypsinization at 37°C was employed in all subsequent studies. An average of 5 × 10⁶ viable keratinocytes/mouse is obtained with a plating efficiency of approx. 65–70% (after 2 days).

Epidermal Cell Cultures

Isolated keratinocytes tend to aggregate in suspension yielding incomplete monolayers at day 2. Phase microscope observation of day-1 and -2 cultures demonstrates that 80% of the T-25 flask

surface is covered by dense areas of polygonal cells containing a clearly visible large nucleus. In Fig. 1*a* is presented a phase micrograph of a day-2 culture before medium change. Most of the unattached cells are removed upon medium change. By days 3-4, complete monolayer formation occurs. At days 5-6, a second layer forms over most of the culture. This uppermost layer, when observed by phase microscopy, consists of cells with an extensive internal network of fibrils that give the keratinocytes a "basket weave" pattern.

Culture stratification continues over days 8-11 in culture. At days 12-14, areas of massive "pile up" or clumps developed, as seen in Fig. 1*b*. The three-dimensional aspects of the stratification of the culture are evident in the scanning electron micrographs presented in Fig. 1*c* and *d*. A monolayer of cells with rough surface membranes is seen at day 2 (Fig. 1*c*). By days 10-11 (Fig. 1*d*), a series of layers develop with areas of "rounded" and partially detached cells or cell-like structures, a phenomenon which was observed to be a prerequisite for "desquamation" of the cultures.

If the cells were grown at 37°C instead of the routine 33°C, a spontaneous sloughing of the top cell layers occurred at day 14. Only large, polygonal and flattened cells possessing a clear cytoplasmic area remained, and these were observed to degenerate by days 22-23. No other cell types were present after keratinocyte death. No sloughing occurs if the cultures are grown at 33°C, and intact multilayered cultures are maintained routinely through day 25 and to at least 6-7 wk of age.

Fibroblast Contamination

The dissociation and Ficoll gradient procedures were designed to eliminate fibroblast contamination of the epidermal cultures. To confirm the absence of fibroblasts in the cultures, keratinocyte and fibroblast cultures and mixed cell type cultures were checked for leucine aminopeptidase activity. The mixed cell type cultures contained keratinocytes and fibroblasts in eight ratios of epidermal to dermal cells, ranging from one fibroblast per six epidermal cells to one fibroblast per 2×10^4 epidermal cells.

As reported by Jacquemont and Pruniéras (20), incubation of the fibroblast cultures with substrate resulted in the appearance of a cytoplasmic purplish-red stain and numerous small granules in a majority of the cells (approx. 70-80%). With no

added substrate, only a yellow-tan color resulted. Using the presence of the granules as criterion, we were able to discriminate, in a blind study, one fibroblast per 2×10^4 epidermal cells (day 4 of culture). Incubation of the keratinocyte cultures with the substrate resulted in no observable leucine aminopeptidase activity.

Electron Microscopy

Several time-course electron microscope studies of the cultures were done. As shown in Fig. 2*a*, a day-2 culture consists of 2 cell layers. Desmosomal complexes, which characterize epidermal skin cells (versus fibroblasts), occurred between all the cells comprising the cultures. At day 6 (Fig. 2*b*), the culture consists of four to five cell layers. Large fibrils (cross- and lengthwise), Golgi complex, and electron-opaque amorphous aggregates are present in the uppermost cells. A representative 10-11 day culture is seen in Fig. 2*c*. By this time, the culture is seven to eight cell layers thick. Fibrils are evident in the uppermost layers, and degenerating nuclei (pycnotic) which contain aggregated heterochromatin are seen in the top layer of the culture. Almost all of the culture shows this type of development. Fig. 2*d* is another representative area of a 10- to 11-day-old culture. It is shown at a lower magnification so as to include the uppermost rounded structure which is a highly specialized cell. By this stage of specialization, the keratinocyte consists of a thickened cell membrane containing numerous fibrils and numerous electron-opaque amorphous aggregates. These structures are always present in the day-10 and older cultures and are continuously shed into the medium.

In Fig. 3*a* and *b*, some of these characteristic epidermal cell structures are shown at higher magnification. The desmosomal membranes show thickening of the inner surface (Fig. 3*a*). The cell in Fig. 3*b* contains both cross-sectional and longitudinal fibrils, numerous free ribosomes, and is in contact with a portion of a thickened cell membrane enclosing numerous fibrils (analogous to those seen in Fig. 2*d*). The cultures maintain this multi-layered pattern to day 35-40 of culture, although areas where excessive shedding had occurred are observed.

Histological Stains

The temporal development of epidermal cell stratification and specialization evident by electron microscopy was confirmed by two histological

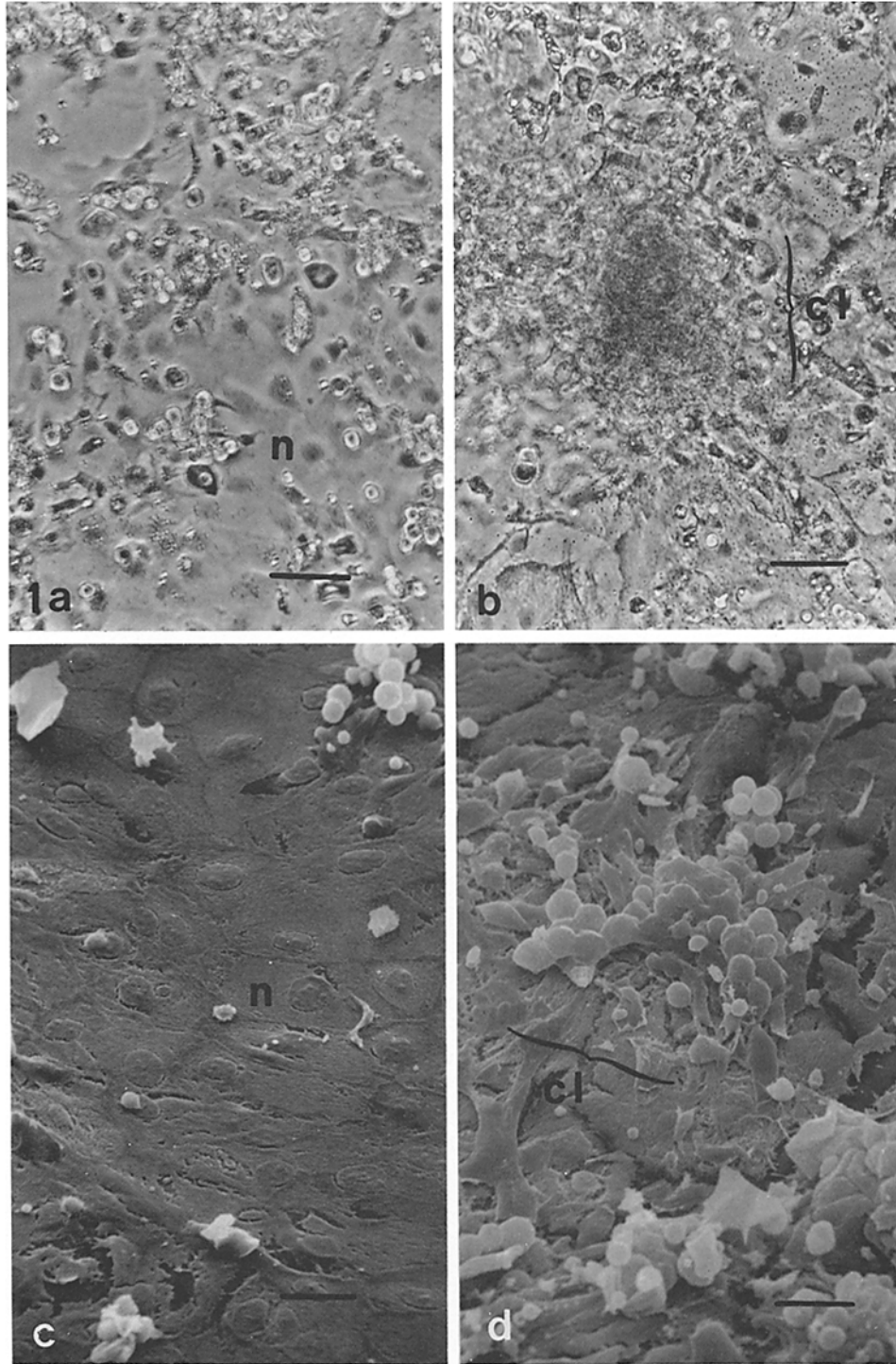


FIGURE 1 Phase and scanning electron micrographs of keratinocyte cultures. (a) Phase micrograph of day-2 culture before medium change. Culture consisted of flat hexagonal cells with prominent nuclei (*n*). Bar, 50 μm . \times 235. (b) Phase micrograph of day-11 culture. Stratification has occurred and areas of cell clumps (*cl*) are evident. Bar, 50 μm . \times 235. (c) Scanning electron micrograph of day-2 culture. The cells forming the monolayer demonstrate rough surfaces and prominent nuclei (*n*). Bar, 25 μm . \times 427. (d) Scanning electron micrograph of day-11 culture. Stratification is evident. Upper layer cells and cell clumps (*cl*) demonstrate smooth cell surfaces. Bar, 25 μm . \times 427.

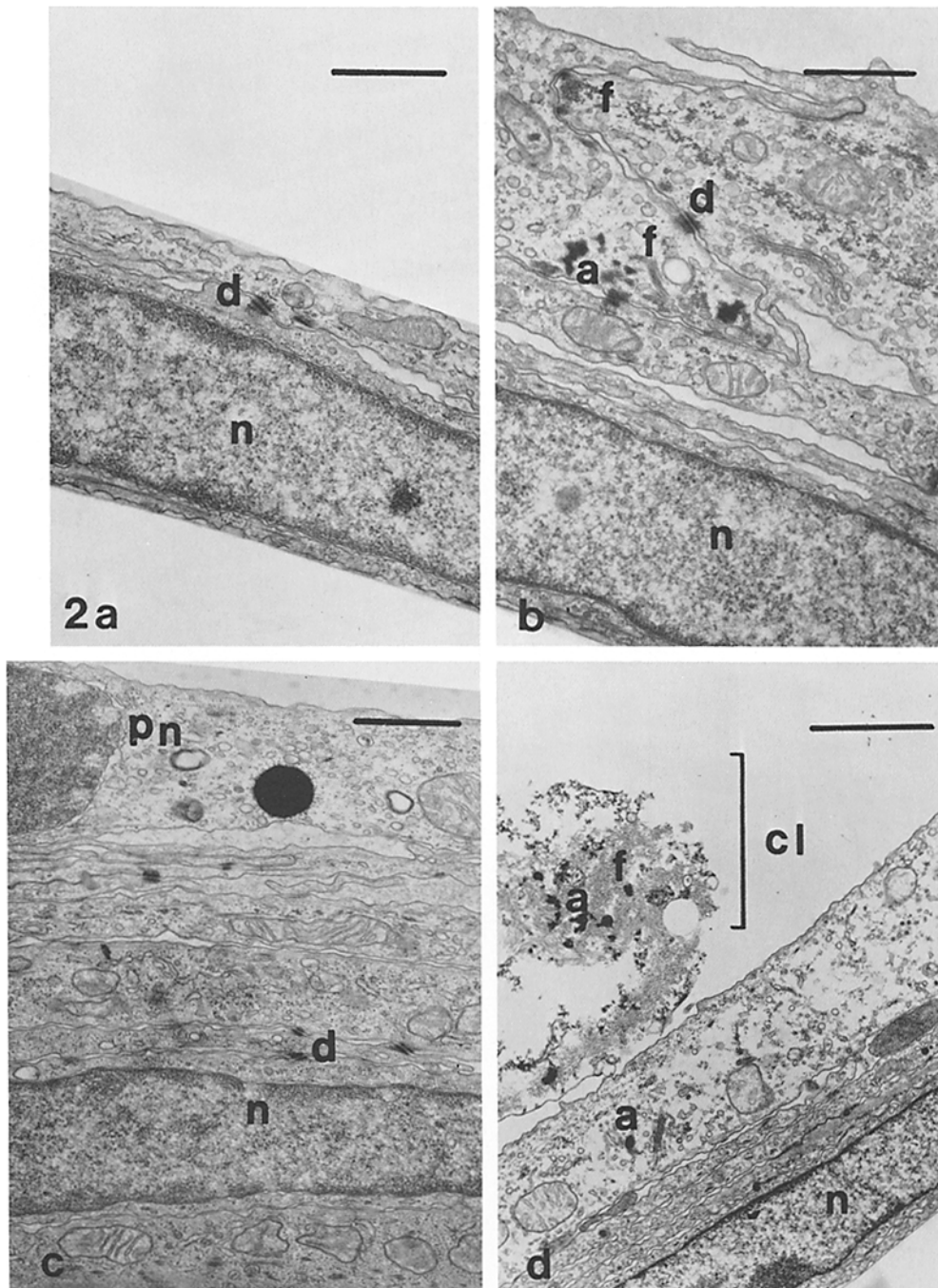


FIGURE 2 Electron micrographs of keratinocyte cultures over time in culture. (a) Day-2 culture consisted of one to two cell layers demonstrating prominent nuclei (*n*) and desmosomal complexes (*d*) at cell-contact points. Bar, 1 μm . $\times 16,000$. (b) Day-6 culture demonstrates four cell layers. The bottom cell layers have prominent nuclei (*n*) and desmosomal complexes (*d*). Fibrils (*f*) and dense amorphous aggregates (*a*) are seen in the upper cell layers. Bar, 1 μm . $\times 14,600$. (c) A day-11 culture consists of six to seven cell layers. Pycnotic nuclei (*pn*) are seen in the upper cell layers. Bar, 1 μm . $\times 14,600$. (d) Day-11 culture demonstrating six cell layers and a clump cell (*cl*) containing amorphous aggregates (*a*) and numerous fibrils (*f*). Bar, 2 μm . $\times 8,000$.

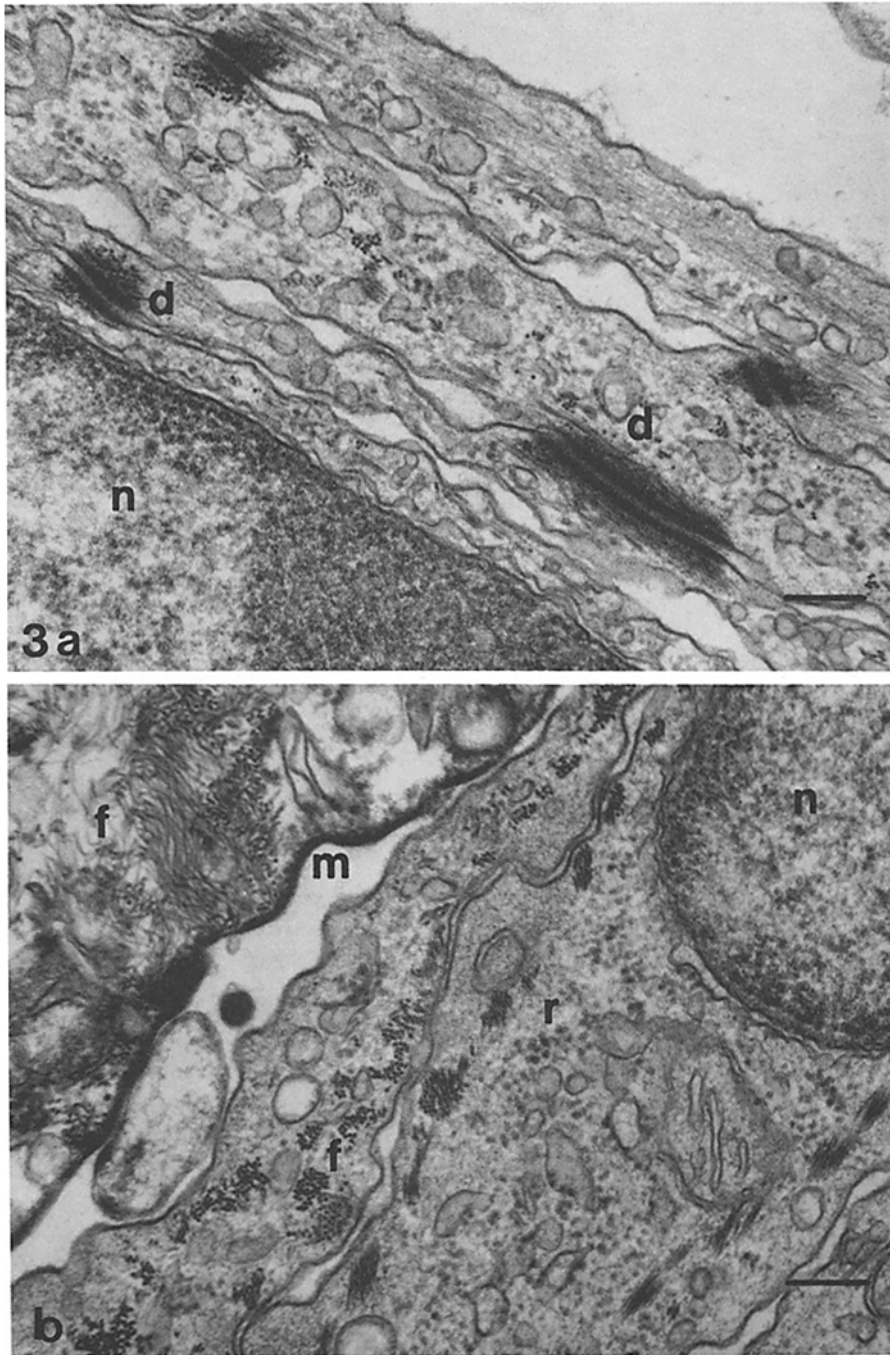


FIGURE 3 Electron micrographs of keratinocyte cultures. (a) Day-7 culture. The desmosomal complexes (*d*) show a thickening of the intercellular contact layers and demonstrate radiating tonofilaments. *n*, Nucleus. Bar, $0.2 \mu\text{m}$. $\times 51,000$. (b) Day-8 culture. The keratinocytes contain cross-sectional and longitudinal fibrils (*f*), numerous free ribosomes (*r*), and are in contact with a portion of a clump cell with a thickened membrane (*m*) that contains numerous fibrils (*f*). *n*, Nucleus. Bar, $0.2 \mu\text{m}$. $\times 51,000$.

stains which have been reported to differentially stain keratin-like material.

In the Kreyberg technique, the basal cell layer of the neonatal mouse epidermis shows only nuclear blue-grey stain. The uppermost layers of the epidermis (spinous, granular, and stratum corneum) stain a vivid red color (erythrosin). The keratinocyte cultures demonstrate the appearance of this staining capacity over time in culture, the intensity being identical to that of the neonatal mouse epidermis. Stained day-2 cell culture shows a layer of polygonal cells, with clear cytoplasm, blue-grey stained nuclei, and light blue-grey cell peripheries. Slight red stain is rarely observed. By days 6-7, when stratification has commenced, the culture contains cells with a blue-grey nuclear stain and a light reddish cytoplasmic stain; some sites of more intense red stain are observed. By day 13 the upper stratified layers exhibit red cytoplasmic staining, and the areas of cell clumps stain a vivid red. In Fig. 4*a-c* are presented micrographs of a stained tissue section and cultures. The areas staining light and dark red are indicated.

With the acid fuchsin-aniline blue-orange G technique, the neonatal mouse skin is defined by a blue-stained basal cell layer (cytoplasm and nuclei) while the upper epidermal cell layers stain with fuchsin. The cell cultures show a stain pattern that varies over time in culture, although the staining quality of the upper neonatal mouse epidermal layers and the upper stratified layers of the cultures are not identical. The staining quality with this technique varied according to fixative, i.e., only Zenker's fixative yielded the reported results, suggesting a required specific interaction between fixative and tissue protein charge groups. It is possible that an *in vitro* variable, i.e., medium components, results in the development of specialized cells with a slightly different fixation and staining quality.

With both staining techniques, day-35 to -40 cultures showed total presence of deeply red staining cell structures. Very flat cells with nuclei (some pycnotic) were discernible in underlying areas. This indicated that, while by phase microscopy the cultures appear unchanged from days 14-20 to days 35-40, in actuality the 6-wk-old cultures consist almost entirely of specialized cells. Electron microscopy of the 35- to 45-day-old cultures demonstrated that the majority of the keratinocytes possess thickened membranes, large

numbers of intracellular fibrils, and poorly formed or absent organelles.

DNA and Protein Content, and Proliferation Characteristics of the Cultures

During the initial days of growth (up to day 4), the keratinocyte cultures contain approx. 40-50 μ g of DNA/flask and demonstrate an increase in Lowry assayable protein (1.0 to 1.7 mg/flask) (Fig. 5). As stratification to 4 to 5 cell layers occurs and specialized keratinocyte characteristics develop (i.e., fibrils, pycnotic nuclei), the cultures accumulate keratinaceous proteins that are not assayable since they are insoluble, and a decrease in DNA content is seen as progressive degeneration of the nuclei in the specializing upper layers occurs.

As culture stratification and specialization continues, the levels of DNA and protein in the cultures remain stable until days 14-17 of growth. This occurs because, during this growth period, the cultures shed the upper cell layers as the bottommost cells proliferate. The sloughed material can be pelleted by centrifugation at 123,000 g (Beckman ultracentrifuge, Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 1 h, and the material does not contain any 3% PCA-hydrolyzable DNA, a characteristic of the upper corneal layer of *in vivo* skin. After the initiation of stratification, the culture as a whole seems to be in a proliferation, specialization, and shedding "steady state" until approximately day 17 of growth. After day 17, the shedding proceeds at an accelerated rate, and it is possible to dislodge the upper cell layers by rough mechanical handling of the cultures.

The change in keratinocyte DNA content and Lowry assayable protein present in Fig. 5 mirrors the specialization of the keratinocytes: an initial high level of DNA and assayable protein is present in the basal cells that comprise the early monolayer; a decrease at days 4-5 occurs as a stable multilayered culture that is specializing and shedding is attained; and at day 17, accelerated shedding and the fragility of the upper layers results in a slight downward shift of the total DNA and protein content.

The DNA synthesis pattern of the cultures over a 23- to 25-day time period was examined. Paired T-flasks from the same experimental series used

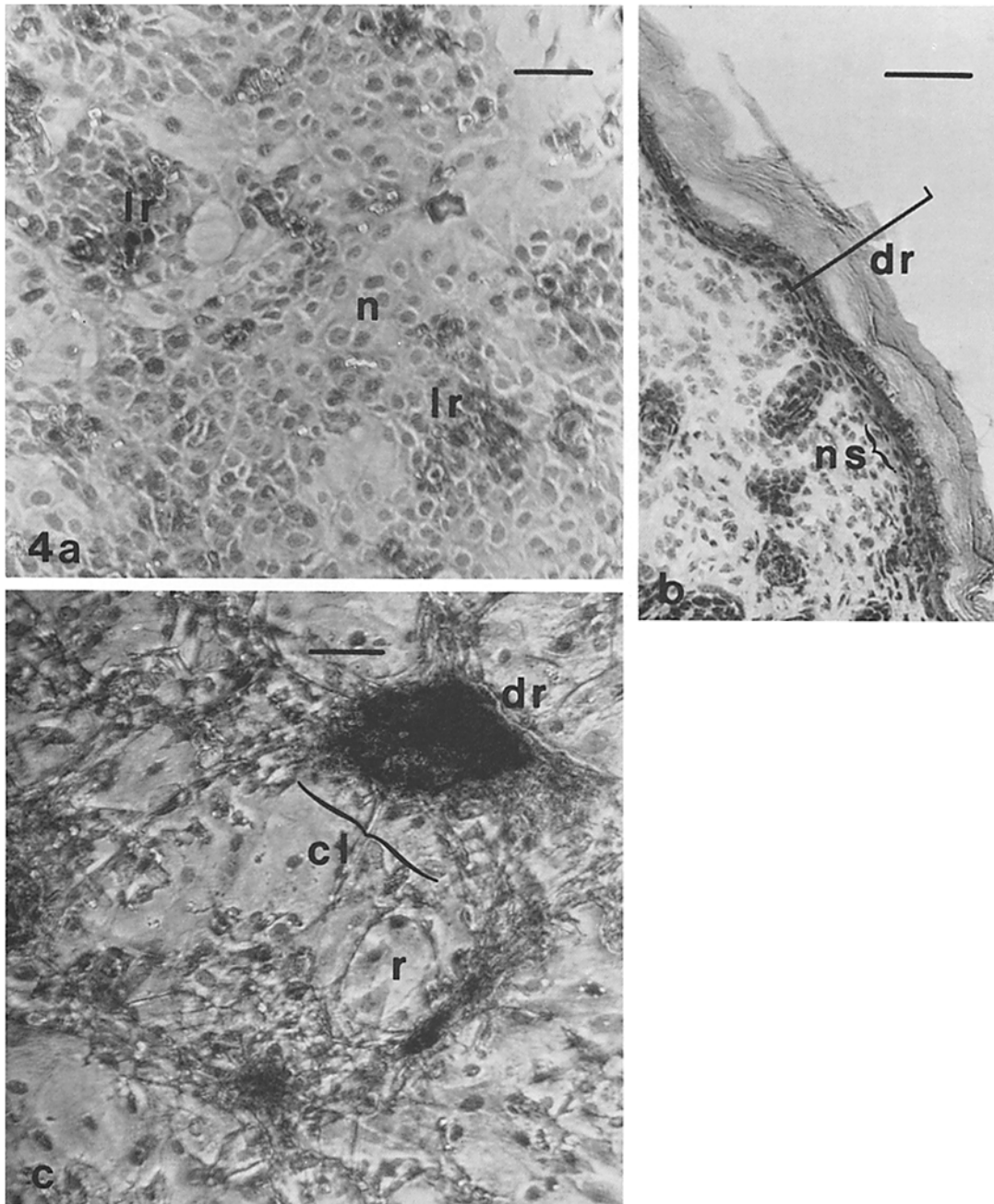


FIGURE 4 Staining pattern of neonatal mouse skin and keratinocyte cultures employing the Kreyberg technique. (a) Day-2 culture. The nuclei (*n*) stained blue-grey, and scattered areas of light red stain (*lr*) occurred. Bar, 50 μm . \times 235. (b) Section of neonatal mouse skin. The spinous, granular, and corneal layers stained dark red (*dr*). The basal layer demonstrated no red stain (*ns*). Bar, 50 μm . \times 243. (c) Day-13 culture. The cell clumps (*cl*) stained dark red (*dr*) while the remaining cell layers showed a red (*r*) staining pattern. Bar, 50 μm . \times 235.

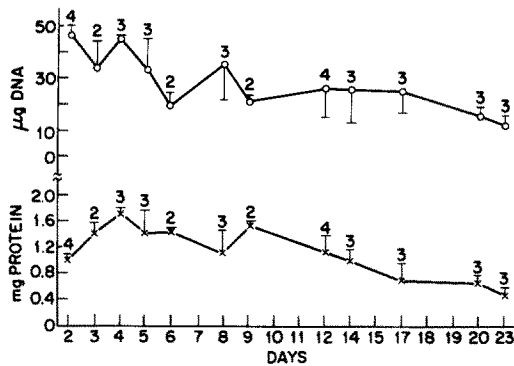


FIGURE 5 DNA and protein content of the cultures over days of growth. At the specified times, quadruplicate flasks were washed and scraped with 6% TCA, and the protein (x) and DNA (O) contents were determined as described in the text. The values are the mean of two to four experiments \pm SEM.

in the DNA and protein study (Fig. 5) were terminally labeled for 4 h with 1 μ Ci/ml of [3 H]Tdr (5–10 Ci/mmol). The DNA was isolated and the quantity of 3 H label incorporated into DNA was counted. The results from three experiments, each spanning a 23-day period, are presented in Fig. 6. Also shown in Fig. 6 is the labeling index (percent labeled nuclei per 500 cells) over the same time frame (days 2–25), using cultures from four other experimental series. As presented in Fig. 6, an oscillating pattern of DNA synthesis was observed with both methods of determining cell proliferation. Quantitation of 3 H label incorporated into DNA by scintillation counting indicated that day-8 and -17 cultures demonstrate the lowest rate of proliferation, and that the greatest DNA synthesis occurs initially and at days 20 and 23. The labeling index of the cultures demonstrates the same pattern of cell proliferation during the initial (until day 8) and final days (17–25) of culture. During the intermediate time span, i.e., days 10–14, culture stratification renders autoradiographic visualization of the labeled low stratum cells impossible. These lower cell layers could be seen to contain labeled nuclei in areas where upper layers were damaged by the fixation process. By days 17–25, sloughing of the upper layers by mechanically shaking the cover slips in BSS and the fixatives allowed more accurate enumeration of labeled nuclei.

The data from six different time-course studies using two methods for analysis of cell proliferation indicated that the cultures demonstrate an oscillating semi-synchronous pattern of cell proliferation.

Moreover, the autoradiographic study showed that 10–30% of the keratinocytes are proliferating during any 20-h time period from days 2–25 of growth. This indicates the presence of a consistent and large number of proliferative stem cells in the cultures.

The activity of the deoxythymidine salvage pathway was examined over the 23-day growth period. To do this, the acid-soluble fraction (6% TCA supernate) was ether extracted and the deoxythymidine nucleoside and nucleotides were fractionated using Dowex 1 \times 2 chromatography. The results indicate that the total radioactive label in the TCA supernate remains relatively constant (6,500–9,000 cpm/flask) over the 23-day growth period. This suggests that no large changes in [3 H]Tdr transport into the cells occurs as the keratinocytes grow in culture. The radioactive label in the acid-soluble fraction was present in the [3 H]Tdr and [3 H]dTTP fractions. From days 2 to 17, the tritium label in the acid-soluble fraction

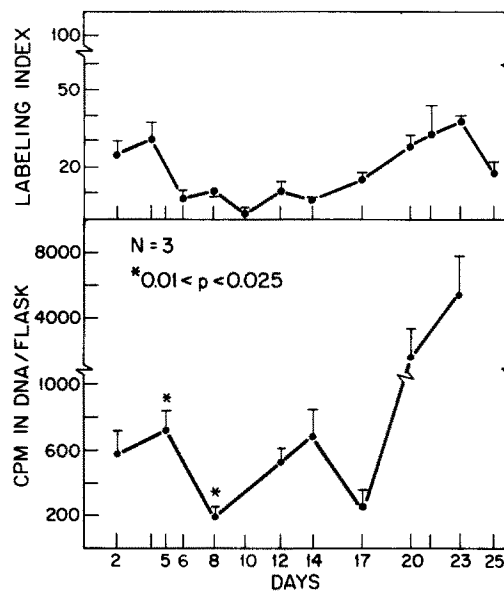


FIGURE 6 Incorporation of [3 H]Tdr into DNA by the cultures over days of growth. Bottom panel: Flasks were terminally labeled (4 h) with 1 μ Ci/ml of [3 H]Tdr (sp act = 5–6 Ci/mmol). Flasks were washed and scraped with 6% TCA as described in the text. The points are the mean of three experiments \pm SEM. * = paired Student's *t* test. Top panel: Flasks containing three Lux plastic cover slips were labeled with 1 μ Ci/ml of [3 H]Tdr (sp act = 60 Ci/mmol) for 18–20 h. The cover slips were washed, fixed, and developed for autoradiography. The points are the mean \pm SEM of from two to four experiments, each lasting for 25 days.

was 2.5–4.0% [^3H]dTTP. On days 20 and 23, slightly higher labeling of [^3H]dTTP occurred (7.75 and 6.75%, respectively), suggesting that a shift in the activity of the deoxythymidine nucleotide salvage pathway accompanied the increase in [^3H]Tdr incorporation into DNA that is observed at days 20 and 23. These data indicate that these parameters, i.e., cpm into DNA/flask, cpm of label in the acid-soluble fractions, percent conversion of [^3H]Tdr into [^3H]TTP and the labeling index, remain relatively constant in cultures derived from a number of different keratinocyte preparations, thus defining the reproducibility of keratinocyte culture proliferative patterns.

Because the usual indicators of culture growth, i.e., increases in cell number, DNA or protein, could not be demonstrated in a continually shedding culture system, it was necessary to demonstrate that [^3H]Tdr incorporation into DNA was a

reliable indicator of replicative DNA synthesis and not a repair phenomenon of a specialized nonreplicating population of plated cells. As presented in Fig. 7 (panel A), the rate of [^3H]Tdr incorporation into DNA over 12 h of terminal labeling on days 3, 9, and 12 was a linear function. Classical inhibitors of DNA synthesis (4°C temperature and $20\ \mu\text{M}$ Ara-C) inhibited the uptake of tritium into DNA, and $<15\%$ of the label was found in the RNA fraction (Fig. 7, panel B). This demonstrates that incorporation of label does not result from nonspecific binding of [^3H]Tdr to cellular DNA or exchange of labeled nucleotide with cellular DNA or RNA.

$2\ \text{mM}$ hydroxyurea, which inhibits replicative DNA synthesis but not DNA repair phenomenon (5), inhibited the incorporation of label into DNA (Fig. 7, panel B), and autoradiography of 20-h, terminally labeled cultures (days 2 and 25) dem-

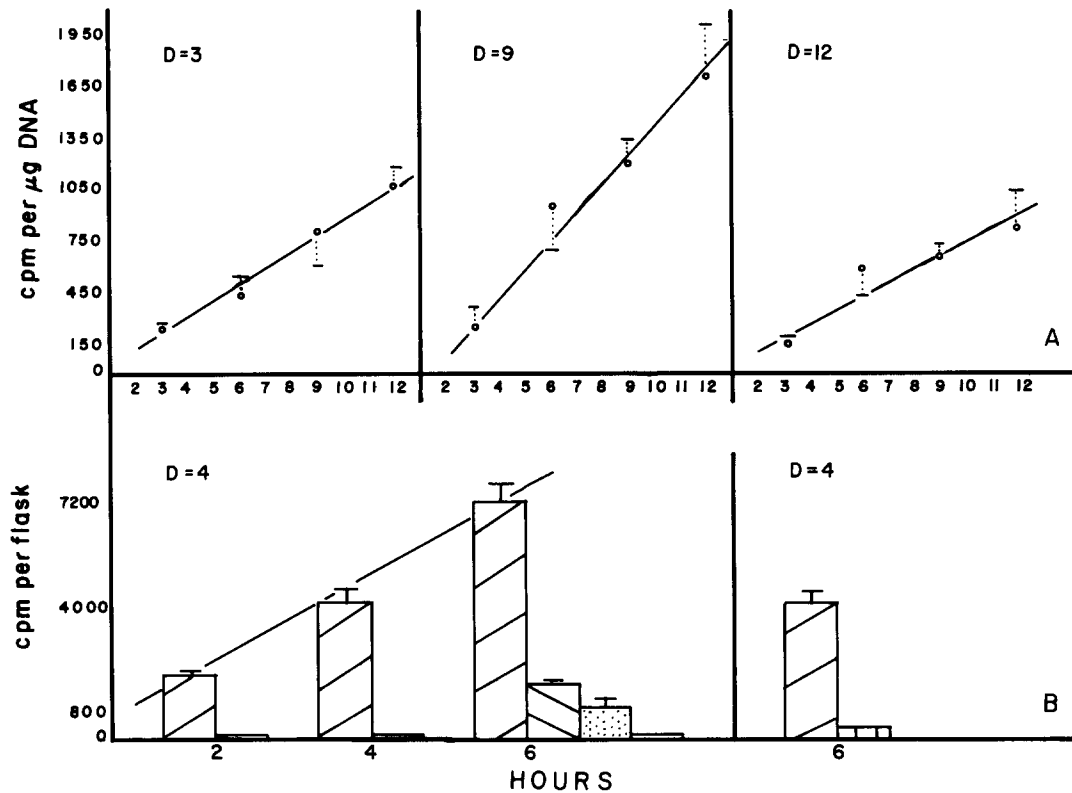


FIGURE 7 Panel A: Linearity of [^3H]Tdr incorporation into DNA over 12 h of terminal labeling with $1\ \mu\text{Ci}/\text{ml}$ (sp act $60\ \text{Ci}/\text{mmol}$) on days (D) 3, 9, and 12 of culture. Each point is the mean of quadruplicate determinations \pm SEM. Panel B: Effect of inhibitors of DNA synthesis on incorporation of [^3H]Tdr into DNA and incorporation of label into RNA (\boxtimes). Drugs and [^3H]Tdr ($1\ \mu\text{Ci}/\text{ml}$; sp act $60\ \text{Ci}/\text{mmol}$) were added at time = 0 h on day 4 of culture. \boxtimes = control; \square = ice; \boxminus = $20\ \mu\text{M}$ Ara-C; \boxplus = $2\ \text{mM}$ hydroxyurea. Each point is the mean of quadruplicate determinations \pm SEM.

onstrated heavy labeling of 10–30% of the nuclei. These data demonstrate that [³H]Tdr incorporation reflects replicative DNA synthesis and not repair.

DISCUSSION

The available trypsinization techniques for isolating the epidermis from the dermal skin component yield a population of keratinocytes that can be maintained *in vitro* for a limited period of time (6, 19, 23) or result in cultures of dedifferentiated passageable keratinocytes (13, 34, 35). These previously defined culture systems have shown some degree of fibroblast co-cultivation (22, 32, 44) as do explant cultures (22, 33), and have been reported to require fibroblast-culture enriched medium (31) or co-cultivation with dermal fibroblasts (23) for optimal culture maintenance.

The primary keratinocyte culture system described in this report shows no detectable dermal fibroblast contamination and requires minimal undefined medium additives in order for highly reproducible entire culture stratification and cell specialization and proliferation patterns to occur over a 3- to 4-wk culture period. Mild dissociation procedures that cause minimal injury to the cells were designed since injury to the skin increases basal cell proliferation (18), and severe dissociation trauma could result in abnormal keratinocyte cell growth patterns. Moreover, excessively injured cells may demonstrate abnormal specialization patterns, a situation that could render *in vitro* investigations of the role of intracellular mediators in keratinocyte physiology difficult or misleading.

In experiments designed to determine optimum dissociation procedures, overnight trypsinization of skin at 4°C as reported by Yuspa and Harris (44) was tried. The cell suspension, after Ficoll gradient purification, consisted of clumps containing 20–30 cells which exhibited more trypan blue dye uptake than cells exposed to trypsin for 1 h at 37°C. These keratinocytes also demonstrated very rapid initial proliferation when plated at low densities and failed to develop any extensive whole culture stratification and specialization. Instead, the cultures consisted of birefringent central cell clumps with sheets of cells surrounding the central aggregates (10, 44). When cells were plated at high densities (5×10^6 cells/T-25 flasks), the flasks showed significant cell death, possibly due to depletion of medium components by the proliferating cell populations. The details of this continuous proliferation pattern of keratinocytes result-

ing from overnight trypsinization procedures have been reported by Elgjo et al. (10).

Our investigation of keratinocyte proliferation did not demonstrate any excessive keratinocyte proliferative pattern during initial culture growth when compared to days 12, 14, or 17 cultures, although increased labeling index and [³H]Tdr uptake into DNA were seen in fully stratified 20-, 23- and 25-day-old cultures. Moreover, cyclic AMP and cyclic GMP radioimmunoassays of partially purified fractions of cultures from the same experimental series presented in Figs. 5 and 6 showed no variation in the cyclic nucleotide content of the cultures over the 23-day period (cyclic AMP = 4.5–6.0 pmol/flask; cyclic GMP = 0.38–0.43 pmol/flask [$n = 3$]; C. L. Marcelo, unpublished results). This suggests that the mechanisms involved in directing the semisynchronous oscillatory pattern of proliferation by these cells do not involve large or chronic changes in either cyclic AMP or cyclic GMP levels. However, the involvement of cyclic nucleotides in pathological epidermal states has been postulated since cyclic nucleotide levels are perturbed in the psoriatic tissue which demonstrates chronic, excessive proliferation and abnormal specialization (28).

Fusenig (15) and Fusenig and Worst (17) have described a neonatal mouse primary epidermal cell system similar in morphology and growth patterns to the one described in this report. However, the dissociation procedures and the growth temperature differ from those used in our system. Briefly, Fusenig (15) and Fusenig and Worst (17) trypsinized minced, full-thickness neonatal mouse skin in a specially designed trypsinization apparatus (trypsinator) that automatically agitates, removes cells, and adds fresh trypsin. The cells, derived from both the epidermal and dermal layers, are isolated by use of two discontinuous Ficoll gradients. Use of the trypsinator and the two gradients seems to cause a decrease in the yield of viable keratinocytes/mouse and results in cells with a plating efficiency lower than that of those obtained with the technique described in this report. Fusenig and co-worker grow the keratinocytes at 37°C, and they report that at this temperature the cultures slough off the top stratified layers, leaving large, flattened cells which degenerate by days 22–25. We have confirmed this observation and have found that culture growth at 32°–33°C, the reported *in situ* temperature of skin (39), extends the *in vitro* life span of fully stratified cultures to day 25, with no decrease

in culture proliferative capacity (29).

By use of anti-mouse epidermis serum in a mixed hemadsorption (43) and immunofluorescence technique (17), Fusenig and Worst verified the epidermal purity of the isolated basal cells. Employing antibodies to pemphigoid antigen (a basal cell-basement membrane junction protein) and to pemphigus antigen (marker of specialized keratinocyte outer cell surface), the status of our culture system has likewise been verified (7). Our studies have indicated that the keratinocytes can, during the first several days of culture, demonstrate pemphigoid antigen and, later in the growth period, develop intense, specific immunofluorescent staining with pemphigoid antibodies.

Phase, transmission and scanning electron microscopy and two histological stains for specialized keratinocyte products demonstrate that the keratinocyte cultures originate as one to two layers of basal cells and proceed to undergo a series of specialization phenomenon resembling in vivo epidermal development. Use of the leucine aminopeptidase technique indicates that there is less than one fibroblast per 2×10^4 epidermal cells in the cultures. The presence throughout the entire monolayer of desmosomal complexes, numerous free ribosomes, and electron-opaque aggregates (probably keratohyalin granules) and the development of uppermost layer cells with thickened membranes containing numerous fibrils indicate that these cultures are epidermal in origin and exhibit an ordered and organized in vitro specialization over a 3- to 4-wk culture period.

As with other primary culture systems, these keratinocytes have limited in vitro lifespan. The loss of cells with a proliferative capacity by 6 wk may indicate that the neonatal cells are developing into a state resembling that of adult epidermis, a tissue with a very limited plating efficiency and in vitro growth potential (16; C. L. Marcelo, unpublished work). The work of Rheinwald and Green (37, 38) and Sun and Green (41), using irradiated 3T3 cell feeder layer, demonstrated that neonatal human keratinocytes can maintain in vitro proliferative capacity, possibly by maintenance of a nonadult cell state through keratinocyte interaction with macromolecular factor(s) particular to 3T3 cells, an established line of embryonic origin (42). The uniqueness of this interaction is attested to by the fact that co-cultivation of keratinocytes and fibroblasts on collagen gels or growth with fibroblast-conditioned medium has failed to significantly extend keratinocyte in vitro lifespan (23,

31; C. L. Marcelo, unpublished results). In the irradiated 3T3-human keratinocyte system, the presence of exogenous cell influences does not allow a clear-cut definition of the role of intracellular mediators in keratinocyte cell function or the effects of drugs or other additives on keratinocytes, i.e., is the observed phenomenon due solely to the epidermal cell, or to the presence of the co-cultured cells, or to some aspect of the interaction of the two cell types? A combination of the neonatal mouse and 3T3-neonatal human keratinocyte systems could be a powerful investigative tool. Initial experiments defining keratinocyte physiology can be conducted on the highly reproducible isolated neonatal mouse keratinocyte system. The results, defining control mechanisms of keratinocyte proliferation and specialized functions, can be concomitantly extended to the heterogeneous human keratinocyte cell system.

This culture system is presently being employed to investigate the role of cyclic AMP and cyclic GMP in epidermal cell function, to delineate events involved in the coordination of cell cycles, and to study the effects of agents known to alter epidermal cell cyclic AMP levels. Details of the effects of cyclic AMP analogue, cholera toxin, and triamcinolone acetonide (30), singly and in combination, on keratinocyte proliferative patterns as well as on intracellular cyclic AMP and cyclic GMP levels will be the subject of subsequent reports.

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