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DIBUTYRYL CYCLIC AMP-INDUCED DIFFERENTIATION OF EPIDERMAL CELLS IN TISSUE CULTURE

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Histochemical and biochemical techniques have been used to compare the effects of dibutyryl cyclic AMP on epidermal cells and dermal cells in primary tissue culture. Rhodamin B staining showed only scattered positive cells in nontreated epidermal cells and a few contaminating keratinizing cell foci in both nontreated and treated dermal cell cultures. In contrast, treated epidermal cells stained strongly and had many keratinizing cell foci. A significant increase in histidine, cystine, and arginine incorporation was noted in epidermal cells treated with dibutyryl cyclic AMP as compared to untreated epidermal cells and to dermal cell cultures both treated and untreated. Dibutyryl cyclic AMP had no significant effect on leucine and phenylalanine incorporation. These results seem to suggest that the intracellular level of cyclic AMP not only controls the synthesis of DNA by epidermal cells in culture but also induces the process of differentiation toward keratinization.

Epidermal cells of adult mammals do not undergo differentiation (keratinization) in primary cell culture [1–5]. Differentiation can be induced by physical and/or chemical changes in the tissue culture conditions [6–8]. The addition of N^6,O^2-dibutyryl cyclic AMP to cell cultures has been shown to induce specific differentiation such as axon formation in neuroblastoma cells [9,10], pigment production in melanoma cells [11], collagen and sulfated acid mucopolysaccharide synthesis in transformed fibroblasts [12,13], and accini formation in thyroid cells [14].

The purpose of this report is to describe the effects of dibutyryl cyclic AMP on adult guinea-pig epidermal and dermal cells. Rhodamin B staining was used to monitor keratinization histochemically [15]. The measurement of radioactivity incorporated into proteins after incubation with ^{3}H-amino acids was employed to determine the rate of synthesis of specific proteins considered to be related to epidermal cell differentiation [16–25].

MATERIALS AND METHODS

Tissue Culture

Two separate primary cultures, one consisting of epidermal cells and the other mostly of dermal cells, were initiated from the ears of 129 Hartley adult guinea pigs according to the method of Regnier et al [4]. A day before the culture, the hairs of the ear skin were plucked with depilatory wax. The skin was washed 3 times with saline, once with 70% ethanol, dried, and rubbed with Mycostatin. On the day of the culture, the animals were killed; the ears were removed, washed 3 times with sterile saline, once with 70% ethanol, and dried with sterile gauze. The ears, with strips of Steri-Strip (3M Minnesota) attached to their backs, were placed on a sterile Petri dish and the skin was cut with a Castroviejo electrokeratome set at level 0.3 mm. The pieces of skin, with Steri-Strips attached, were floated on 0.15% trypsin in Hanks' buffer solution (calcium- and magnesium-free), pH 7.2, at 37°C for 90 min. The skin was rinsed in medium (BME Eagle 1955 supplemented with 10% calf serum from Flow Laboratory) and placed with keratinized layers down on a Petri dish. After the dermis was lifted with fine forceps, the epidermal cells on the Steri-Strips were removed by very gentle scraping with a scalpel and put in a tube containing the medium. The dermal tissue was also placed in the same tube and shaken for 30 sec with a Vortex mixer at speed 1 in order to obtain basal cells attached to the dermal surface. The medium, containing epidermal cells in suspension and the dermal tissue, was filtered on sterile gauze placed on the top of a sterile beaker. The epidermal cells obtained in the filtrate were seeded in Petri dishes or in flasks ($\sim 1 \times 10^6$ cells/ml).

The pieces of dermis which remained on the gauze were immersed in 0.1% collagenase (Worthington) in a glucose–potassium–sodium solution according to Hinz and Syverton [26] at 37°C for 90 min, then put in a tube containing medium and dissociated by shaking for 30 sec with a Vortex mixer at speed 1. The dermal cells in suspension obtained after filtration on sterile gauze were seeded in Petri dishes and in flasks ($\sim 1.5 \times 10^5$ cells/ml).

Forty-five Petri dishes (diameter 35 mm, Falcon) and 48 flasks (75 mm^2, Falcon) were established for epidermal cells and 45 Petri dishes and 57 flasks for dermal cells.

Treatment with Dibutyryl Cyclic AMP

At 72 hr after seeding, one-half of the Petri dishes and flasks were incubated with medium containing 10^{-3} M

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dibutyryl cyclic AMP (N^6,O^2-dibutyryl adenosine 3',5'-cyclic monophosphoric acid, Sigma). The control medium and the medium with dibutyryl cyclic AMP were changed every day.

For additional control, 2 flasks each of epidermal and dermal cells were incubated with medium containing 10^{-4} M sodium butyrate.

Microscopy

Cell cultures were observed every day with a phase microscope (Diavert, Leitz). After 240 hr of treatment, 21 Petri dishes containing epidermal cells (10 control and 11 treated) and the same number of Petri dishes containing dermal cells, were washed 3 times with Hanks', fixed with 3% glutaraldehyde for 30 min, postfixed with 2% osmium for 1 hr, and embedded in a mixture of Epon and Araldite. Thick sections were cut, stained with 1% toluidine blue and examined with a Leitz microscope.

Rhodamin B Staining

At 6, 8, and 10 days after treatment, 8 Petri dishes containing epidermal cells (4 controls and 4 treated) and 8 containing dermal cells were washed with Hanks', fixed with 4% formalin for 2 min, stained with 0.1% toluidine blue in aqueous solution for 2 min, followed by 0.1% rhodamin B in aqueous solution for 10 min. The cells were observed under a light microscope and the staining density was measured with a densitometer (Quick Scan Flur Vis, Helena Lab. Corp.). The average density of treated cells was expressed as a percentage of the control.

Incorporation of Tritiated Amino Acids

On the tenth day after treatment, the flasks were incubated for 1 hr with 1 μCi/ml of tritiated histidine, cystine, arginine, leucine, or phenylalanine. The cells were washed 3 times with 10 ml of saline, dissolved in 2 ml 1 N NaOH, and separately transferred to centrifuge tubes. They were neutralized with 2 ml HCl and precipitated by adding 4 ml 1 N perchloric acid (PCA). After centrifugation at 10,000 rpm for 10 min, 0.1 ml of the supernatant was used to determine the amount of radioactivity in the free amino-acid pools using a scintillation cocktail (18 gm PPO, 1 l Triton X-100, 2 l toluene) in a Beckman liquid scintillation counter. The pellets were washed 2 times with 0.5 ml 0.5 N PCA, suspended in 0.5 ml 0.5 N PCA, and hydrolyzed at 95°C for 10 min. The tubes were then placed in ice for 30 min and centrifuged at 13,000 rpm for 20 min. One-tenth of a milliliter of the supernatant was used for determination of radioactivity in PCA-hydrolyzed proteins; 2 other aliquots of 0.1 ml were used to measure the amount of DNA by the diphenylalanine method of Burton [27]. The pellets were hydrolyzed in 2 ml 6 N HCl at 100°C for 24 hr. An aliquot of 1 ml was counted to determine radioactivity of the PCA-insoluble proteins. The total radioactivity was expressed according to the formula:

$$\frac{\text{cpm in 0.5 ml 0.5 N PCA} + \text{cpm in 2 ml 6 N HCl}}{\mu\text{g DNA in 0.5 ml 0.5 N PCA}}$$

The radioactivity in the treated cells was calculated as a percent of the control. The studies were performed with, at least, triplicate samples and repeated twice in the case of tritiated histidine, cystine, and leucine. Incorporation of tritiated arginine was examined in duplicate samples, twice, and that of tritiated phenylalanine was studied once in triplicate samples.

RESULTS

Microscopy

By direct observation with a phase microscope, the primary cultures derived from trypsinized epidermis were composed of sheets of epithelial cells scattered throughout the Petri dishes or the flasks (Fig. 1). Very few branched cells, identifiable as melanocytes, were visible and they sometimes formed bridges between adjacent groups of growing cells. For the first 5 days, the treated epidermal cells looked like the control cells. On the sixth day, the treated epidermal cells showed more discrete borders and they started to form multilayered clumps. These changes increased during the 10 days the treatment was continued (Fig. 2).

Primary cultures, derived from collagenase-treated dermis, were composed mostly of fibroblastic cells (Fig. 3) and a few small epithelial islands. Morphologic changes were noted as early as the third day of treatment; dermal cells became more spindle shaped and contained more grains in the cytoplasm. These changes increased as long as the treatment was continued (Fig. 4). The number and size of the epithelial islands did not appear altered after the treatment.

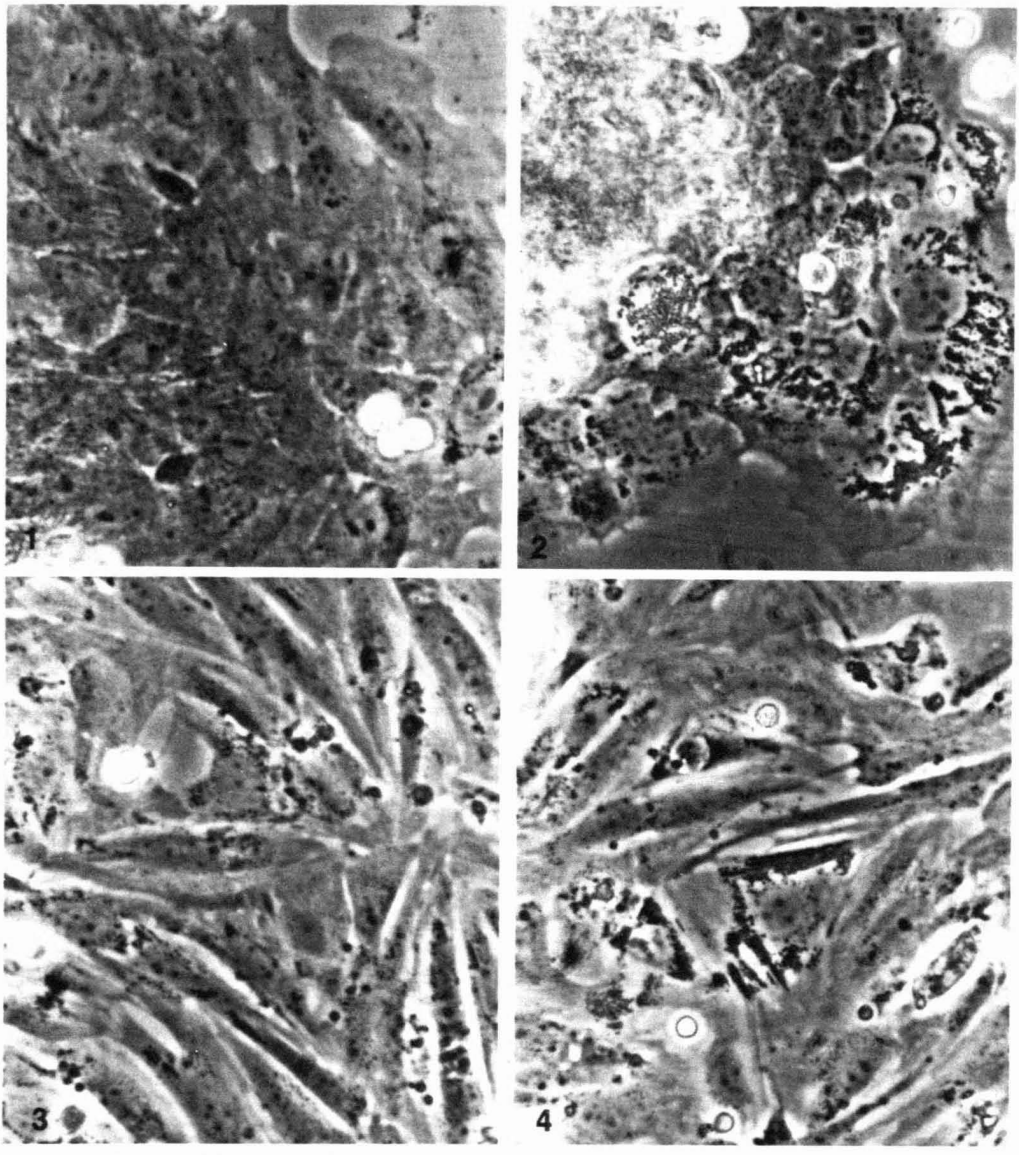
Thick sections of epidermal cells confirmed the formation of multilayer clumps in treated cells. While control cells were flat, the treated cells were round and often increased in volume. Some keratohyaline granule-like structures were observed in the cytoplasm. Cells at the surface of the culture appeared to be cornified (Figs. 5, 6).

Thick sections of dermal cells demonstrated that most cells were very flat and formed a monolayer. Epithelial cell clumps present in the dermal cells consisted of round and keratinizing cells in which keratohyaline granule-like structures were seen. The epithelial cells in the control and treated cultures appeared morphologically identical.

Rhodamin B Staining

In nontreated epidermal cells, individual rhodamin B-positive cells were found scattered through the Petri dish. In both nontreated and treated cultures established from dermis, there were very small foci rather strongly stained with rhodamin B. These keratinizing cells were contaminating epidermal cells. The number of these foci varied from 10 to 20 in the control Petri dishes. No increase in size or number of foci was observed after treatment. In contrast, the treated epidermal cells were strongly stained, containing a large number of keratinizing cells. At 6 days after treatment, there were large, rhodamin B-positive foci through the Petri dishes. After 8 days of treatment, the foci became confluent and almost covered the surface of the Petri dishes (Fig. 7).

The stain densities in the Petri dishes, monitored by densitometer at 6, 8, and 10 days, are summarized in Table I. The stain density increased 2-fold in epidermal cells, whereas it was about the same as the control for dermal cells after



FIGS. 1 and 2. Effect of dibutyryl cyclic AMP on 13-day-old epidermal cells as shown by phase microscopy (× 600). Guinea-pig ear epidermal cells grew as a "epithelial-like" sheet. After addition of DBcAMP into the medium for 10 days, the cells formed multilayered clumps (Fig. 2) as compared to nontreated cells (Fig. 1).

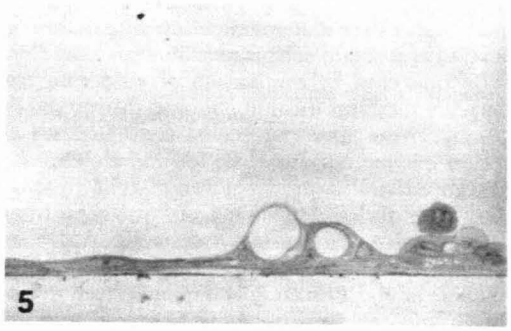
FIGS. 3 and 4. Effect of dibutyryl cyclic AMP on 13-day-old dermal cells as shown by phase microscopy (× 600). Guinea-pig ear dermal cells appeared "fibroblast-like." After 10 days of treatment with DBcAMP, the cells became more spindle shaped and exhibited granules (Fig. 4) as compared to nontreated cells (Fig. 3).

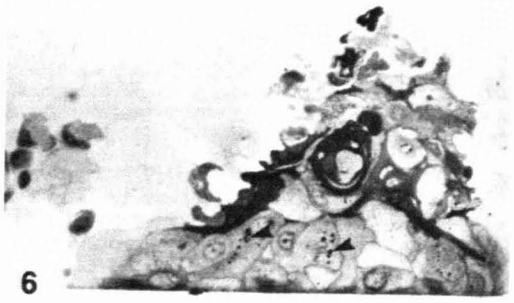
6 days' treatment. After 8 and 10 days' treatment, the stain density increased to more than 3 times as high as the control for epidermal cells, whereas it remained about the same as the control for dermal cells.

Incorporation of Tritiated Amino Acids

The results are summarized in Figure 8. Incorporation of [^{3}H]histidine, [^{3}H]cystine, and [^{3}H]arginine into PCA-soluble and epidermal protein (6 N HCl plus hot PCA hydrolysate) increased 2.6, 4.5, and 3.6 times, respectively, in epidermal cells after treatment, whereas the rate of incorporation of these ^{3}H-amino acids into dermal cells after the treatment was about the same as the nontreated controls. In contrast, the incorporation of [^{3}H]leucine and [^{3}H]phenylalanine was about the same as the control for either epidermal cells or dermal cells.

Table II shows that the specific radioactivity of the acid-soluble fraction was not affected by treatment with dibutyryl cyclic AMP. The pool was also the same after treatment with sodium butyrate. The sodium butyrate did not increase the incorporation of [^{3}H]histidine in epidermal cells.





FIGS. 5 and 6. Effect of dibutyryl cyclic AMP on 13-day-old epidermal cells (1-μ sections) (× 875). Nontreated, control cells grow as a monolayer sheet (Fig. 5). In some areas, cells form 2 or 3 layers. After 10 days of treatment with DBcAMP, the cell clumps consisted of about 10 cell layers and cornification seemed to occur (Fig. 6). Note keratohyaline-like granules (→) present in the cells.

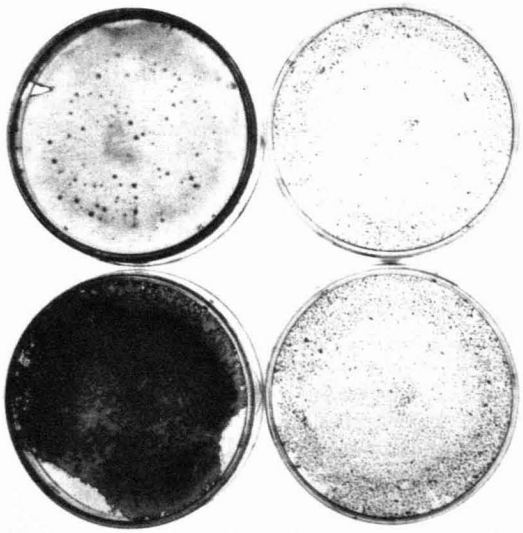
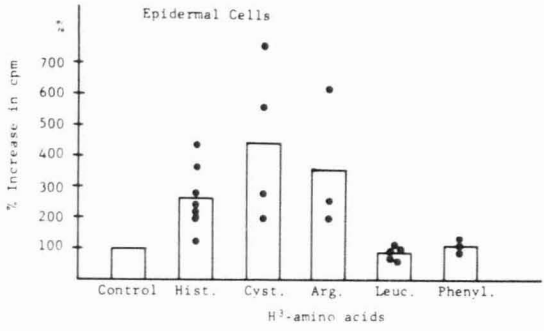


FIG. 7. Effect of dibutyryl cyclic AMP on epidermal cells as shown by rhodamin B staining. Thirteen-day-old cultures of nontreated epidermal cells (*A*), 10-day DBcAMP-treated epidermal cells (*B*), nontreated dermal cells (*C*), and 10-day DBcAMP-treated dermal cells (*D*).

TABLE I. *Rhodamin B staining*

The data are density averages of 4 Petri dishes of nontreated and treated epidermal and dermal cells monitored by densitometer at 6, 8, and 10 days after treatment with dibutyryl cyclic AMP. The density obtained with nontreated cells was taken as 100%.

Days	Epidermal cells	Dermal cells
6	215%	105%
8	335%	111%
10	348%	109%



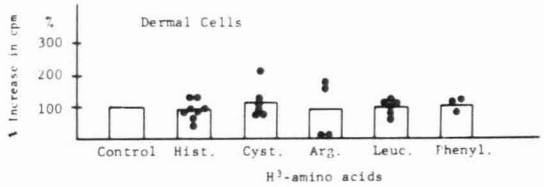


FIG. 8. Effects of 10 days' treatment with dibutyryl cyclic AMP on the incorporation of ^{3}H-amino acids into proteins in 13-day-old epidermal and dermal cell cultures. The rate of protein synthesis found in the control cells was taken as 100% and that in the treated cells was expressed as percent increase.

DISCUSSION

Cells prepared from guinea-pig ear epidermis grew as a sheet and rhodamin B stained only a few scattered cells. As previously reported [1–5], the epidermal cells in the tissue culture condition which we used did not keratinize. Contaminating epidermal cells in the dermal cell cultures grew as small epithelial islands surrounded by other dermal cells, most likely fibroblasts. These epidermal cells, predominantly originated from hair follicles, showed clumping and formed cornified cells which stained with rhodamin B, indicating that they were keratinized as has already been described by Fusenig et al [28]. These findings coincide with those reported by Karasek and Charlton [6], who observed keratinization of rabbit, mouse, and human epithelial cells, grown on collagen gels either alone or containing skin fibroblasts or in media conditioned by skin fibroblasts, and thus suggested the need for dermal factors in growth and differentiation of epithelial cells.

The addition of dibutyryl cyclic AMP caused considerable changes in the morphology of epidermal cells in tissue culture. They became multilayered, and showed keratohyaline granule-like struc-

TABLE II. *Effects of sodium butyrate and dibutyryl cyclic AMP on amino acid pools and protein synthesis*

The effects of 10 days' treatment of sodium butyrate (NaBu) and dibutyryl cyclic AMP (DBcAMP) on the pool of free [^{3}H]histidine and free [^{3}H]phenylalanine and on the amount of these amino acids incorporated into protein are recorded. Thirteen-day-old epidermal and dermal cell cultures were used. cpm obtained from the control cells were taken as 100% and cpm from the treated cells were expressed as percent increase.

	Epidermal cells		Dermal cells	
	NaBu	DBcAMP	NaBu	DBcAMP
Histidine				
Free amino acids	103%	121%	86%	94%
Incorporated amino acids	97%	267%	100%	93%
Phenylalanine				
Free amino acids	71%	67%	91%	108%
Incorporated amino acids	81%	110%	77%	108%

tures as well as cornified cells. These cells were also stained intensely with rhodamin B, a specific histochemical staining for cornified cells in tissue sections [15].

Fukuyama et al [19–22] demonstrated by autoradiography that injected [^{3}H]histidine, [^{3}H]arginine, and [^{3}H]cystine primarily incorporated into proteins of granular cells (differentiated cells) whereas [^{3}H]leucine and [^{3}H]phenylalanine concentrated in basal cells (undifferentiated cells). Furthermore, ultrastructural autoradiography showed that the former amino acids were specifically involved in the formation of keratohyaline granules. We, therefore, used the rate of incorporation of these ^{3}H-amino acids into proteins of epidermal cells in tissue culture as a radioactive marker for the formation of granular or differentiated cells. A great increase in the incorporation of [^{3}H]histidine, [^{3}H]cystine, and [^{3}H]arginine was found in dibutyryl cyclic AMP-treated epidermal cells as compared with that in the control cells. The results were considered indicative of an increase in the formation of specific proteins rather than an increase in the total protein synthesis, since incorporation of [^{3}H]leucine and [^{3}H]phenylalanine was not stimulated by the same treatment. The cells prepared from the dermis did not show changes in the rate of incorporation of any amino acids after the addition of dibutyryl cyclic AMP. Epidermal cells already in the process of differentiation did not seem influenced by the additional dibutyryl cyclic AMP. This view is apparently supported by histochemical and morphologic findings that both the size and number of differentiating epidermal cell islands observed in dermal cell culture remained about the same after treatment.

The precise role played by dibutyryl cyclic AMP in the induction of keratinization in primary dissociated epidermal cells is not known. Constable et al [7] observed keratinization of epidermal cells when the medium was not changed during the first 3 days. These epidermal cells were clumped and formed a layered system. Bauer and De Grood [8] aggregated subcultured epidermal cells from guinea-pig epidermis by the use of "nucleoproteins." It is possible that dibutyryl cyclic AMP may change the adhesion of epidermal cells in culture as Johnson and Pastan [29] demonstrated in fibroblasts, and the keratinization observed may be a secondary effect. Epidermal cells in the dermal cell culture may not have been influenced because they were already in clumps before the dibutyryl cyclic AMP was added.

The present study, however, does not eliminate another possibility that dibutyryl cyclic AMP directly stimulates epidermal keratinization. There are a number of reports concerning the regulation of cellular metabolism by cyclic AMP in vivo and in vitro (e.g., stimulating protein synthesis at transcriptional and/or translational level [30]).

The third possibility is that epidermal cells differentiated as a result of cessation of cell division. Fusenig et al [28] and Christophers [31] showed that differentiation of embryonic mouse hair follicle cells and guinea-pig epidermal cells was decreased when DNA synthesis was stimulated by vitamin A acid. Delescluse et al [32] studied the effects of cyclic AMP-elevating agents in epidermal cell proliferation. They found that dibutyryl cyclic AMP inhibits 50 to 90% of cell growth. Differentiation of the epidermal cells observed in this study may be secondary to the inhibition of cell growth. On the other hand, two different control mechanisms of growth and differentiation may exist in vivo. Stimulation of cell growth was shown to accompany an increase in differentiation after in vivo epidermal cell injury [33] or mitogenic stimulation [25].

In primary dissociated epidermal cells, induction of keratinization by treatment with dibutyryl cyclic AMP occurred repeatedly in all experiments of the present study and an increase in the rate of specific protein synthesis measured was quite consistent. The technique used may provide an experimental model for studies of regulation of gene expression involved in keratinization.

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