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Augmentation of Keratinocyte Differentiation by the Epidermal Mitogen, 8-Bromo-cAMP

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The effect of the epidermal mitogen, 8-bromo-cAMP, on keratinocyte differentiation was studied. A 3×10^{-4} M dose of 8-bromo-cAMP was added to primary neonatal mouse epidermal keratinocyte cultures that slowly proliferate, stratify and differentiate over 2–3 weeks time. [^{3}H]Thymidine autoradiography coupled with an NH_4Cl plus reducing agent technic which separates basal and differentiating keratinocytes was used to determine the target cell for the 8-bromo-cAMP mitogenic effect. A histologic stain and a four buffer protein extraction protocol, in conjunction with PAGE and fluorographic technics, were used to assess the differentiation of the cultures. The data indicated that 8-bromo-cAMP primarily stimulated the proliferation of the basal cell monolayer. Simultaneous with the mitogenic effect was an increase in the production of keratohyalin granule, keratin and cell envelope proteins, which are specific markers of epidermal differentiation. The results indicate that keratinocytes stimulated by the epidermal mitogen 8-bromo-cAMP simultaneously express differentiation-related processes.

The effect of cAMP on proliferation appears to be cell-specific and concentration-dependent. Numerous studies report that large increases in intracellular cAMP inhibit proliferation [1, 2], an event often accompanied by stimulation of a number of cell functions and of differentiation [3–8]. Almost as numerous are investigations demonstrating the hyperproliferative effect of cAMP on a number of cell types [9–14].

cAMP, at low cellular concentrations, is an epidermal keratinocyte mitogen, both in vitro [15, 16] and in vivo [17, 18]. cAMP has also been reported to stimulate epidermal keratinocyte differentiation, at least on a morphologic level [15, 17]. We have used neonatal mouse primary cultures which show a hyperproliferative response to 8-bromo-cAMP [15, 19] to study the effect of cAMP on the production of specific epidermal keratinocyte proteins.

The neonatal mouse keratinocyte cultures start as a basal cell monolayer. The cultures stratify, forming 7–8 cell layers and the cells contain specific epidermal keratinocyte structures, such as desmosomal complexes, keratin filaments and keratohyalin granules. The differentiation proteins forming these structures can be separated into six fractions using a procedure recently described by us [20].

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Table 1. *Epidermal protein fractions of neonatal mouse keratinocyte culture*

Fraction	Designation	Extraction buffer[a]	Comment
High salt-soluble proteins	S_2	1 M KPO_4, pH 7.0	Proteins that remain in solution after extensive dialysis
Keratohyalin proteins	R_2	1 M KPO_4, pH 7.0	Macroaggregated precipitates formed during dialysis
SDS-soluble proteins	S_3	1% SDS, 0.05 M $NaPO_4$, pH 7.0	SDS soluble proteins from non-enveloped cells
Non-disulfide-cross-linked keratin	S_4	4 M urea, 0.05 M $NaPO_4$, pH 7.0	Labelled by a brief exposure to [^{3}H]amino acids
Disulfide-cross-linked keratin	S_5	8 M urea, 0.1 M 2-mercapto-ethanol, 0.001 M DL-dithio-threitol, 0.05 M $NaPO_4$, pH 7.0	Labelled only after a long chase period
Envelope proteins	R_5	8 M urea, 0.1 M 2-mercapto-ethanol, 0.001 M DL-dithio-threitol, 1% SDS, 0.05 M $NaPO_4$, pH 7.0	Insoluble, high MW structure; does not enter 5% acrylamide gel

[a] All extraction buffers were supplemented with 10 μg/ml PMSF, a proteinase inhibitor.

Using SDS polyacrylamide gel electrophoresis (SDS-PAGE) and fluorographic technics we have studied the effect of a mitogenic dose of 8-bromo-cAMP on the production by epidermal keratinocytes of specific epidermal proteins. Our results indicated that 8-bromo-cAMP stimulated both epidermal keratinocyte proliferation and differentiation. Thus, epidermal keratinocyte cultures can express differentiation-associated processes while actively proliferating.

MATERIALS AND METHODS

Reagents and Animals

Day-old neonatal BALB/c mice were bred in a colony maintained at the University of Michigan, Department of Dermatology. Medium 199 (modified, Earle's salts, glutamine), fetal bovine serum (FBS) and other medium components, were purchased from Flow Laboratories, Rockville, MD. Ultrapure urea was from Schwarz–Mann, Spring Valley, N.Y. 8-Bromoadenosine 3′:5′-cyclic monophosphate, sodium salt (8-bromo-cAMP) and 8-bromoguanosine 3′:5′-cyclic monophosphate, Na salt (8-bromo-cGMP), were obtained from Sigma Chemical Co., St Louis, Mo.

All materials for electrophoresis were purchased from Bio-Rad Laboratories, Richmond, Calif. Phenyl methylsulfonyl fluoride (PMSF) was from Calbiochem (San Diego, Calif.). All other chemicals used were obtained from Matheson, Coleman and Bell, East Rutherford, N.J. L-[4,5-^{3}H]Leucine, 170 Ci/mmol, 1 mCi/ml was purchased from Radiochemical Centre, Amersham, UK. [Methyl-^{3}H]-thymidine, 78.2 Ci/mmol, 1 mCi/ml and EN^{3}HANCE were purchased from New England Nuclear, Boston, Mass. Corning plastic products (Corning, N.Y.) were used in all phases of cell isolation and growth. Nuclear Track photographic emulsion (NTB) and X-Omat X-ray film were obtained from Kodak, Rochester, N.Y.

Epidermal Keratinocyte Cultures

Primary keratinocyte cultures were prepared from neonatal mouse skin as previously described [19]. 5×10^6 viable basal cells were plated in 60 mm Petri dishes and grown in medium 199 supplemented with 13% (v/v) FBS plus 50 IU/ml of penicillin and 50 μg/ml streptomycin. The cultures were grown at 32°C under 5% CO_2 humidified gassing, and were fed every other day.

Kreyberg Staining of Keratinocyte Cultures

The Kreyberg stain was used to differentiate basal cells (cytoplasm and nuclei stain blue) from suprabasal, and differentiated cells which stain a red erythrosin color [19].

Addition of Cyclic Nucleotide Analogs

Twenty four hours after plating 3×10^{-4}M 8-bromo-cAMP was added to the cultures and with each medium change.

Measurement of DNA Synthesis

Neonatal mouse keratinocyte cultures have very minimal de novo synthesis of DNA precursors [21], so that [^{3}H]thymidine labelling of DNA via the salvage pathway is a reliable index of DNA synthesis and of cell proliferation in these cells. Cultures grown on coverslips were labelled for 16 h at 32°C with 10 μCi/ml of [^{3}H]thymidine and autoradiography was done as previously described [19]. The data is expressed as per cent positive cells ± SEM.

Separation of Basal and Differentiating Keratinocytes

The keratinocytes grow as multi-layered cultures [19]. To separate the proliferating basal from the differentiating keratinocytes, cultures growing on coverslips were rinsed 2x with PBS and then incubated in a solution of 0.24 M NH_4Cl, 0.1 M 2-mercaptoethanol, 0.25 M sucrose, pH 9.0 either for 30 min or for 16 h at 4°C. After mechanical agitation, the differentiating keratinocytes were dislodged leaving the basal cells attached to the coverslips. The detached keratinocytes were squashed on a slide and both cell preparations were fixed. Kreyberg staining and autoradiography were then done on the cells.

Extraction of [^{3}H]Leucine-labelled Proteins from Keratinocyte Cultures

After a 2 h pulse with 10 μCi/ml [^{3}H]leucine, five extraction buffers are used in series to separate the cell proteins into six fractions [20]. The buffers and the type of protein in each fraction are summarized in table 1.

Quantitation of [^{3}H]Leucine and of the Protein in the Fractions

To compare control with treated cultures, we used the only parameter that does not change as the keratinocytes are affected by additives such as cAMP: the unit of one Petri dish containing an initial culture of 5×10^6 cells [20]. The data is expressed as: (*a*) Total μg protein per dish or culture; (*b*) total cpm of [^{3}H]leucine per dish or culture; (*c*) total μg protein or cpm [^{3}H]leucine in a fraction per dish or culture.

SDS–PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and fluorography (SDS–PAGE) was done as described by Laemmli [22] and as previously reported by us [20]. Equal portions of a culture were run for each fraction, i.e., S_3 protein extracted from ¼ Petri dish of a 2-day-old control and of an 8-bromo-cAMP-treated culture was run. The intensities of the protein bands thus allow a visual comparison of the polypeptides. For fluorography, the gels were impregnated with EN^{3}HANCE, dried and were exposed to X-Omat x-ray film at −70°C for 1–2 weeks.

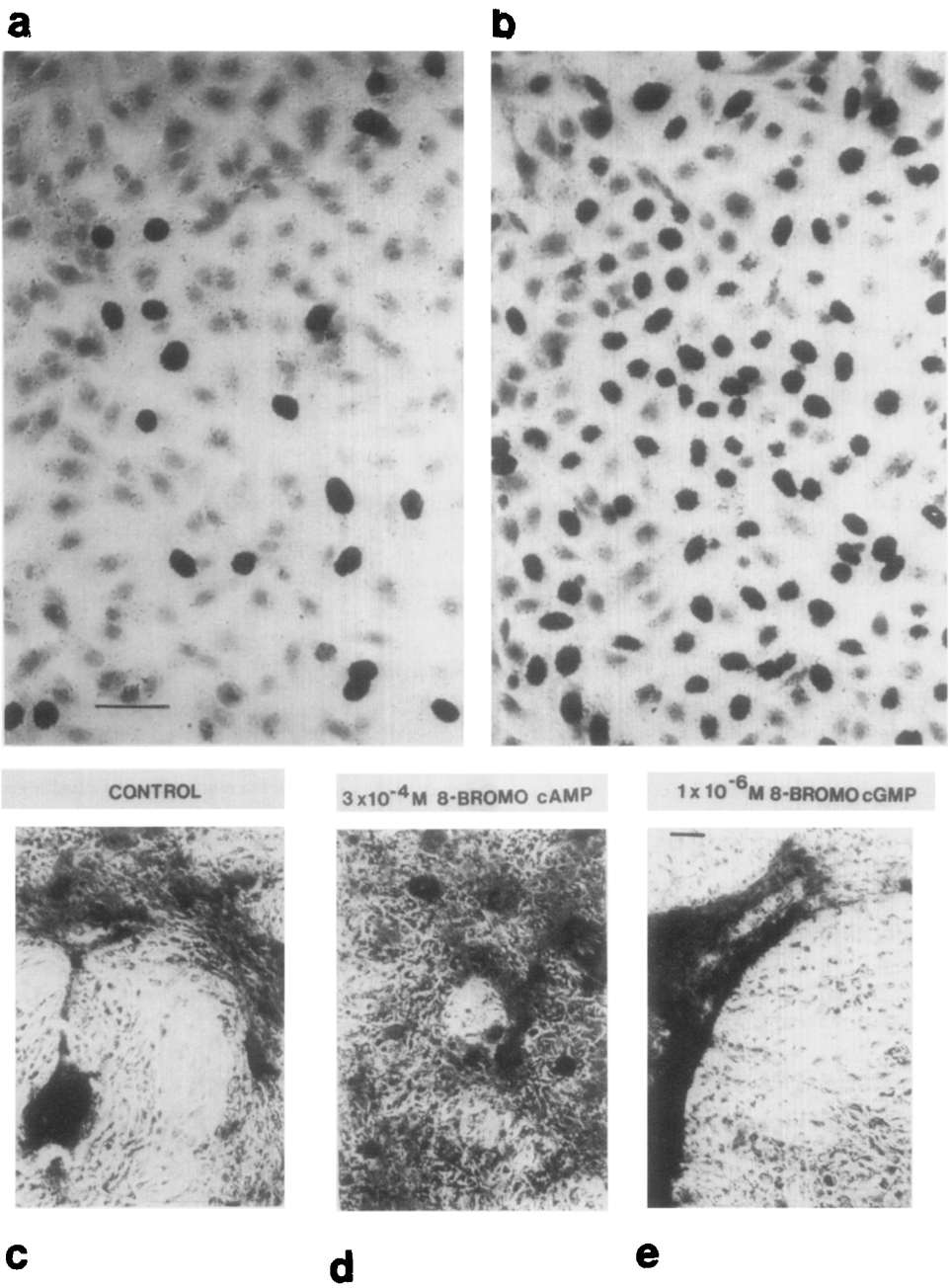

Fig. 1. Autoradiograms of basal keratinocytes and integrity of differentiating cell layers from control and cyclic nucleotide treated cultures. (*a, b*) 16-day-old cultures were pulsed with 10 μCi/ml [^{3}H]thymidine for 16 h and the differentiating layers were removed using an NH_4Cl+2-mercaptoethanol solution. (*a*) Control: 4.3%±0.8 labelling index; (*b*) 3×10^{-4} M 8-bromo-cAMP: 50%±4 labelling index. (*c, d, e*) Kreyberg-stained 12-day-old control cultures. 10^{-6} M 8-bromo-cGMP was used as an additional control; the effect is specific to cAMP. Bar, (*a, b*) 45 μm; (*c–e*) 60 μm. (*a, b*) ×225; (*c–e*) ×85.

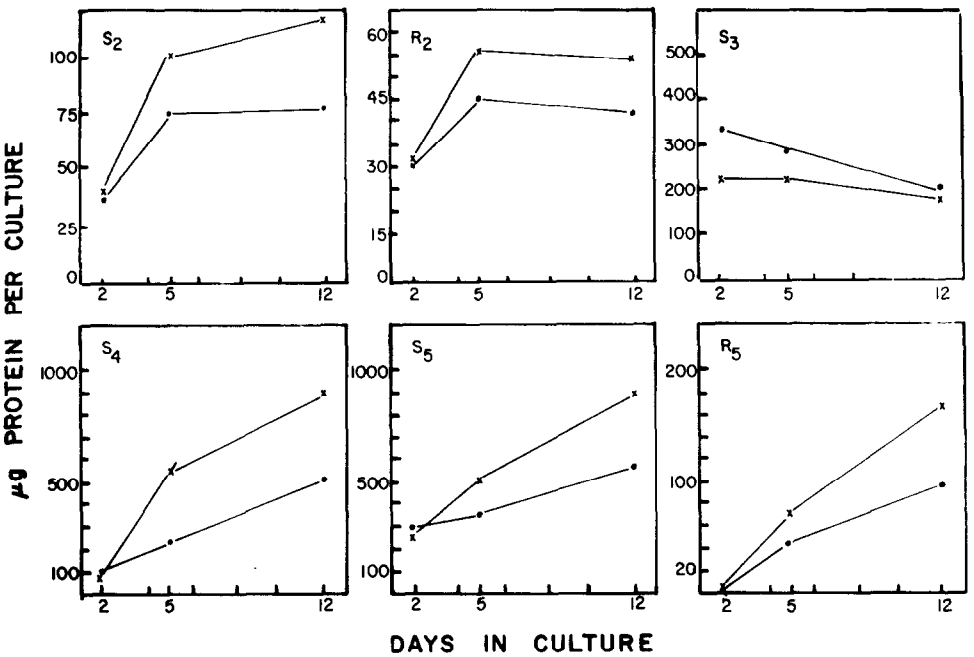

Fig. 2. Protein fractions extracted from control, and 8-bromo-cAMP-treated cultures. 2-, 5- and 12-day-old control and 3×10^{-4} M 8-bromo-cAMP-treated cultures were serially extracted and the total protein per fraction was determined. S_2, High salt soluble proteins; R_2, keratohyalin granule-associated proteins; S_3, SDS-soluble proteins from viable, non-enveloped keratinocytes; S_4, non-covalently cross-linked keratins; S_5, disulfide cross-linked keratins; R_5, cell envelope proteins. ●——●, Control cultures; ×——×, 3×10^{-4} M 8-bromo-cAMP added.

RESULTS

Effect of 8-Bromo-cAMP on [^{3}H]Thymidine Labelling of Basal Keratinocytes

Autoradiograms of control and 8-bromo-cAMP-treated epidermal keratinocyte cultures after removal of the differentiating cell layers are shown in fig. 1 *a, b*. The basal cell layer from the 16-day-old control cultures showed 4.3%±0.8 labelling of the cells. The cultures grown in 3×10^{-4} M 8-bromo-cAMP had a 50%±4 labelling index. Some [^{3}H]thymidine-labelled cells (estimated at 2–3% of the population) were seen in differentiating cell preparations from both the control and 8-bromo-cAMP-treated cultures.

Effect of 8-Bromo-cAMP on the Integrity of the Stratifying Cell Layers

12-Day-old keratinocyte cultures show areas of detachment and of sloughing of fully differentiated cornified cells (fig. 1 *c*). 8-Bromo-cAMP-treated cultures retained the integrity of the upper cell layers (fig. 1 *d*, 12-day-old) for as along as 20 days in culture (data not shown). Cultures treated with 8-bromo-cGMP, at its active dose of 10^{-6} M, were morphologically identical to the control cultures (fig. 1 *e*), indicating that the effect was specific to cGMP.

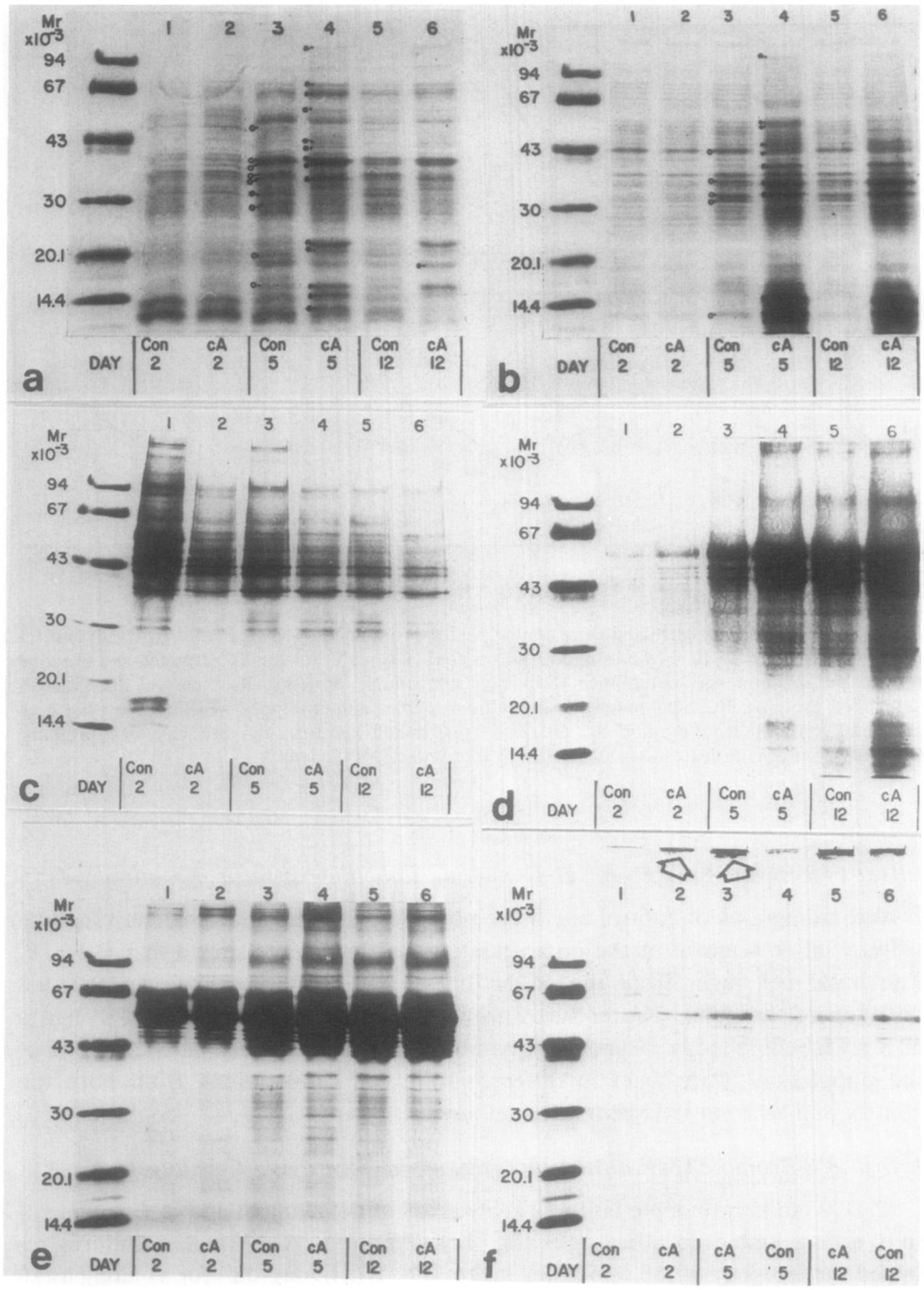

Fig. 3. Coomassie blue-stained SDS PAGE of control and 8-bromo-cAMP-treated cultures. 2-day-old basal cell, 5-day-old stratifying and 12-day-old fully differentiated keratinocytes were pulse labelled for 2 h with 10 μCi/ml [^{3}H]leucine and extracted. Ten one-time-point experiments were done with the

Table 2. *Effect of 8-bromo-cAMP on total protein and [^{3}H]leucine incorporation in keratinocyte cultures*

Days after plating[a]	Control	8-Bromo-cAMP	Change (%)
	μg protein/culture		
2	805	531	34 ↓
5	1 039	1 536	48 ↑
12	1 501	2 218	48 ↑
	cpm of [^{3}H]leucine/culture		
2	935 991	821 421	12 –
5	540 321	765 851	42 ↑
12	347 445	447 445	29 ↑

[a] 3×10^{-4} M 8-bromo-cAMP was added 1 day after plating, thus the cultures were grown for 1, 4 and 11 days in the cyclic nucleotide.

Ten 60 mm Petri dishes per time point were scraped and combined to yield enough protein for this and other analyses [20]. There was no change in the uptake of [^{3}H]leucine by the 2-, 5- or 12-day-old 8-bromo-cAMP-treated cultures, as determined by scintillation counting of the acid-soluble (6% TCA) pools of parallel cultures.

Analysis of the Proteins Extracted from Control and cAMP-Treated Cultures

Cultures treated with 3×10^{-4} M 8-bromo-cAMP and control cultures were pulse labelled on day 2, 5 and 12 with 10 μCi/ml of [^{3}H]leucine.

The quantities of total protein/culture and total cpm [^{3}H]leucine/culture are presented in table 2. The amount of protein approximately doubled as the control keratinocytes grew from a basal cell monolayer to a fully differentiated 12-day-old culture. An initial decrease in the amount of total protein in the cultures, possibly resulting from the immediate cAMP mitogenic effect, or an effect on plating efficiency, was seen. This was probably not cytotoxic, since these proliferating cultures showed an increase in total protein of 48% in the stratifying (day 5) and 48% in the fully formed cultures (day 12). As the cultures grew, the amount of [^{3}H]leucine incorporated into protein by the control culture cells decreased, as we have previously reported [20]. 8-Bromo-cAMP increased by 42% (day 5) and 29% (day 12) the amount of [^{3}H]leucine label in the proteins.

In fig. 2, the amount of protein in the control and 8-bromo-cAMP-treated cultures per fraction is presented. As the control basal cell cultures stratified, the amount of high salt soluble (S_2) and of keratohyalin granule macroaggregated

2-day to 12-day time period. 8-Bromo-cAMP invariably induced the same effect presented in this figure, fig. 2 and table 2. (*a*) S_2, High salt extractable proteins; (*b*) R_2, keratohyalin granule macroaggregates; (*c*) S_3, SDS-soluble, viable cell proteins; (*d*) S_4, non-covalently-cross-linked keratins; (*e*) S_5, disulfide-cross-linked keratins; (*f*) R_5, cell envelopes. Polypeptide band ●, intensity increased by 8-bromo-cAMP; ⁎, apparently induced by 8-bromo-cAMP; ○, in control cultures not present in 8-bromo-treated cultures.

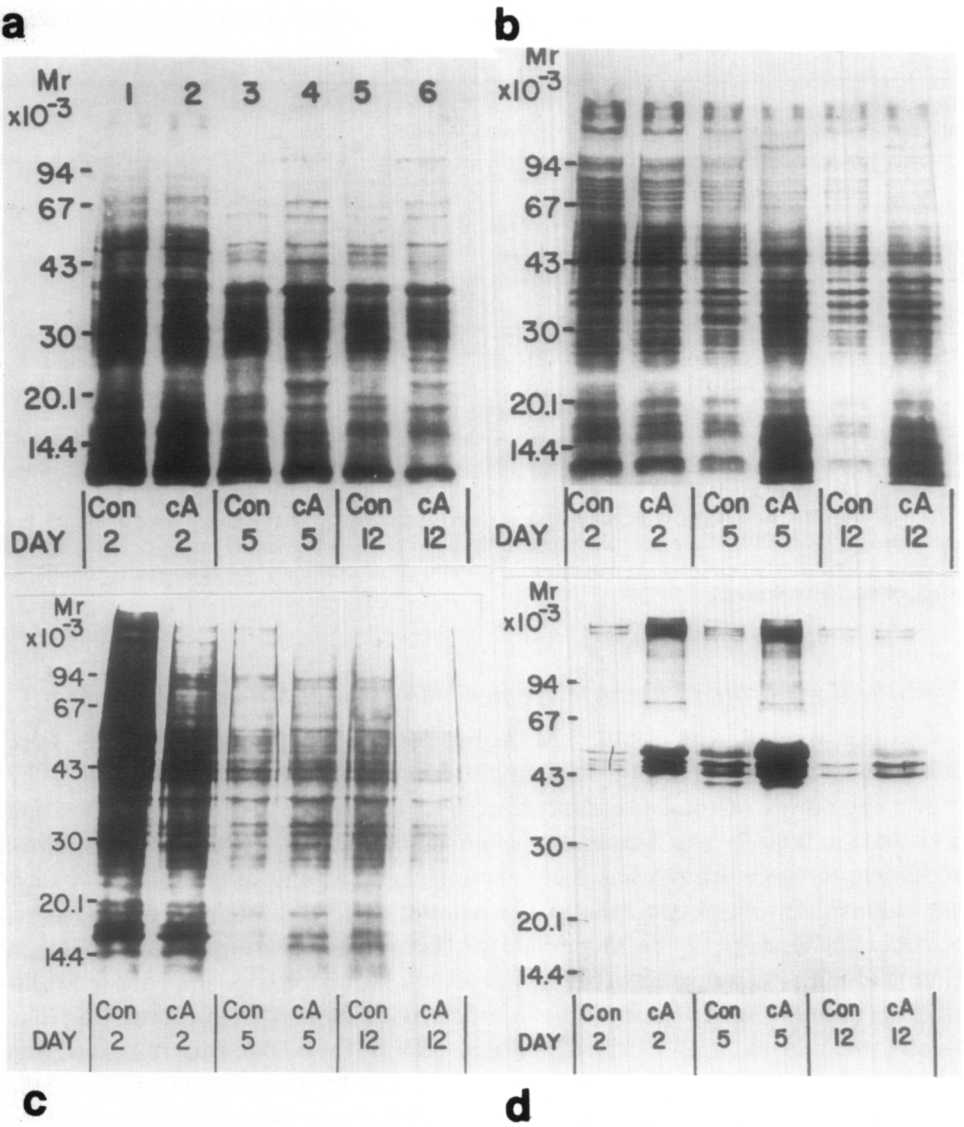

Fig. 4. Fluorograms of SDS–PAGE of control and 8-bromo-cAMP-treated cultures. (*a*) S_2, high salt-extractable proteins; (*b*) R_2, keratohyalin granule macroaggregates; (*c*) S_3, viable cell proteins; (*d*) S_4, non-covalently cross-linked keratins. The S_5, disulfide cross-linked keratins, and cell envelope proteins (R_5) from control cultures are not labelled during the 4 h hot and cold chase time period. 8-Bromo-cAMP treatment did not alter this observation.

(R_2) proteins approximately doubled (day 5). The non-cross-linked keratins (S_4), the disulfide-cross-linked keratins (S_5) and the cell envelope proteins rapidly increased, by as much as 5-fold, as the control basal cell cultures differentiated. Concomitantly, the amount of viable cell, SDS-soluble protein (S_3) decreased slightly by day 5, and by approx. 30% in the fully differentiated cultures.

8-Bromo-cAMP stimulated the production of the keratohyalin granule S_2 and R_2 proteins 1.5- to 2-fold and of the keratins (S_4 and S_5), and of the cell envelope proteins (R_5) approx. 2- to 3-fold over the amounts of protein in the control cultures. The quantity of S_3 viable cell proteins was decreased approx. 30% by 8-bromo-cAMP on day 2 and 5 of growth.

SDS–PAGE and Fluorography of Proteins from Control and 8-Bromo-cAMP-Treated Keratinocyte Cultures

Coomassie blue-stained slab gels of proteins extracted from control and 8-bromo-cAMP-treated cultures are presented in fig. 3. All the fractions extracted from 5-day-old and 12-day-old 8-bromo-cAMP-treated cultures, except for S_3, contained more protein than the control cultures. The amount of a number of S_2 and R_2 polypeptides was increased by 8-bromo-cAMP treatment (fig. 3 *a* and *b*, lane 4, solid circles). 8-Bromo-cAMP also apparently induced the synthesis of a number of other polypeptides, marked by asterisks in S_2 (fig. 3 *a*, lane 4) and R_2 (fig. 3 *b*, lane 4). The protein in a number of other bands extracted from 5-day-old 8-bromo-cAMP-treated cultures was decreased when compared to the controls; these are indicated by open circles on the no. 3 control lanes (fig. 3 *a, b*). Within the limits of SDS PAGE resolution and visual scrutiny, fifteen bands in S_2 and eleven bands in R_2 were stimulated or induced by 8-bromo-cAMP, and nine S_2 bands and eleven R_2 polypeptides were inhibited by this cAMP analog.

The non-cross-linked keratin fractions (S_4) contains seven keratin polypeptides, ranging in MW from 42.7 to 57.6 kD ([20], fig. 3 *d*). No difference in the polypeptide composition between the control and 8-bromo-cAMP-treated cultures was observed (fig. 3 *d*). The 12-day-old control cultures contain much more non-cross-linked keratin protein than the 2-day-old basal cell cultures [20]. Much larger amounts of these keratins were found in the 8-bromo-cAMP-treated keratinocytes (fig. 3 *d*). 8-Bromo-cAMP also increased the amounts of the disulfide-cross-linked keratins in the keratinocyte cultures (fig. 3 *e*). The cell envelope proteins (R_5) do not even enter the stacking gel [20]. However, the amount of these proteins were also observed to be increased in 5-day-old 8-bromo-cAMP-treated cultures (arrow) (fig. 3 *f*).

As presented in fig. 2, the SDS-soluble proteins (S_3) decreased in amount as 2-day-old cultures stratified and differentiated (fig. 3 *c*). The 8-bromo-cAMP-treated cells, especially the 2-day-old cultures, contained significantly less protein than the control cultures.

Fluorograms of the protein fractions are presented in fig. 4. 8-Bromo-cAMP increased the labelling of the R_2 keratohyalin granule macroaggregate fraction of 5-day-old and 12-day-old cultures, whereas no difference in [^{3}H]leucine labelling of the S_2 proteins was seen. The S_4 non-disulfide-cross-linked keratins extracted from the 8-bromo-cAMP-treated cultures also showed increased incorporation of the [^{3}H]leucine label, especially by 2-day-old and 5-day-old cultures. As seen in

fig. 3*c*, less intensely labelled bands were present in S_3 cell soluble protein from the 8-bromo-cAMP-treated keratinocytes.

DISCUSSION

The epidermis is a tissue in dynamic equilibrium, i.e., the differentiated keratinocytes are continuously lost through shedding and are replaced by cells produced in the proliferative basal cell layer [23]. Cultured epidermal keratinocytes likewise proliferate, maintaining a basal cell monolayer which supplies cells to the differentiating cell layers [19].

Our work and that of a number of other investigators demonstrate that stimulation of the "second-messenger" cAMP system induces epidermal keratinocyte proliferation, both in vitro [15, 16] and in vivo [17, 18]. We present in this report evidence that the basal cell monolayer is the target cell for the cAMP mitogenic signal.

8-Bromo-cAMP specifically caused a greater than 10-fold increase in the number of [^{3}H]thymidine-labelled cells in the basal cell layer of the cultures. Some ^{3}H-labelled cells were seen in the differentiating cell layers. These are either a small number of non-basal cells that, in vitro, maintained their proliferative capacity, or as suggested by Lavkar & Sun [25], are ^{3}H-labelled basal keratinocytes which were vertically displaced after mitosis. Specific antibody staining of these cells show they are basal epidermal keratinocytes [24].

Keratinocyte cultures undergoing cAMP-induced hyperproliferation show enhanced overall culture stratification and differentiation, as seen in histologic and electron morphologic studies [15]. Our present studies showed that 8-bromo-cAMP increased the synthesis of keratohyalin granule-associated proteins and of keratin polypeptides. The amounts of all these proteins associated with epidermal differentiation [26–30] were induced by growing the cultures in a mitogenic dose of 8-bromo-cAMP.

The cAMP-induced increase in the production of these proteins occurred 4 days after adding the cAMP although the mitogenic effect of 8-bromo-cAMP occurs at earlier time points [15]. cAMP specifically has this effect: 8-Bromo-5'AMP did not induce the morphologic changes [15] and 10^{-6} M 8-bromo-cGMP caused no consistent change in the epidermal keratinocyte proteins [15].

8-Bromo-cAMP apparently induced the expression of (fig. 3, asterisk) and increased the amounts of (fig. 3, point) a number of polypeptides extracted into the keratohyalin granule-associated protein fractions (S_2+R_2). This cAMP effect occurred in only these two fractions and the function of these induced polypeptides is at present unknown.

Other studies by us have concentrated on cAMP-dependent protein kinase phosphorylation of these same epidermal keratinocyte protein, since this is the only known mode of cAMP action [31]. We found that 8-bromo-cAMP induced ^{32}P-labelling of acidic proteins in the S_2 keratohyalin granule fraction [32].

Studies by Lonsdale-Eccles, Haugen & Dale [33] and Steinert et al. [30] have shown that a specific epidermal differentiation protein, named filaggrin (a histidine-rich protein), undergoes phosphorylation and dephosphorylation prior to integration into the stratum corneum, the uppermost epidermal layer. The 8-bromo-cAMP-induced polypeptides in the S_2 fraction could be either polypeptide markers of differentiation, or mediators of cAMP action.

Disulfide cross-linking of keratin polypeptides is part of the epidermal differentiation program [34]. The studies reported here and our other preliminary studies [32] showed that 8-bromo-cAMP did not affect this process, although very small differences beyond the detection limits of our technology may be occurring.

Growth in 8-bromo-cAMP also specifically stabilized the integrity of the differentiating cell layers (fig. 1 *c, d, e*). The greater stability of the upper layers could easily result from an increased number of cells forming a better and tighter upper layer, i.e., a better "barrier" is produced. Alternatively, 8-bromo-cAMP may induce greater quantities of intercellular cementing substances, or may stimulate the formation of desmosomal complexes and cell junctions which are involved in the binding of epidermal keratinocyte cells [35].

The results presented in this report allow us to make the following conclusions.

(1) cAMP has a mitogenic effect primarily (only?) on the epidermal basal cell.

(2) The 8-bromo-cAMP-activated basal cells show accelerated proliferation. The progeny are most probably other basal cells and suprabasal cells capable of differentiating.

(3) There is no evidence that 8-bromo-cAMP increases the synthesis per differentiating cell of keratins, cell envelopes and other differentiation marker proteins, although some polypeptides directly related to the mode of action of cAMP have been detected. 8-Bromo-cAMP appears to simply increase the number of differentiating cells per culture.

Thus the data supports the hypothesis that 8-bromo-cAMP stimulation of epidermal keratinocyte proliferation and differentiation results only from a mitogen signal on the basal cell. Other epidermal keratinocyte mitogens, i.e., the tumor promotor tetradecanoyl phorbol acetate (Marcelo, unpublished data) and growth in low Ca^{2+} medium [36, 37], have different effects. These two mitogens significantly decrease epidermal differentiation. How the cAMP mitogen signal differs from the TPA and low Ca^{2+} mitogen effect on epidermal differentiation is presently being studied.

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