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CONTROL OF KERATINOCYTE DIVISION *IN VITRO**

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ABSTRACT

Aqueous extracts of newborn rat epidermis inhibit mitosis of cultivated keratinocytes obtained from guinea pigs and humans. The mitotic activity of cultivated human keratinocytes and fibroblasts obtained from the uninvolved skin of subjects with psoriasis is similar to the mitotic activity of these cells obtained from involved psoriatic skin. This observation is consistent with the model of repression of mitotic activity in normal skin and a derepression of mitotic activity in psoriatic epidermis. Extracts of liver also inhibit mitosis of guinea pig keratinocytes. Cultivated keratinocytes do not exhibit the same specificity of inhibition by epidermal extracts that has been reported for surviving fragments of mouse ear.

Psoriasis is a genetically determined disease characterized by scaly red plaques most commonly located on extensor surfaces and regions of trauma. The clinical abnormalities of psoriasis reflect alterations in the cytokinetics of the psoriatic keratinocyte. The duration of the basal epidermal cell cycle in a psoriatic lesion is abbreviated (1, 2, 3) and the time spent by the basal keratinocyte in transit to the stratum corneum is also decreased (3, 4). A tenable model of psoriasis is one in which the normal control of epidermal cell division is lost; therefore factors influencing basal cell mitosis have been investigated.

The epidermis consists of three populations of cells:

1. stem cells (cells capable of synthesizing DNA)
2. differentiating cells (Malpighian and granular cells)
3. differentiated cells (stratum corneum)

In a psoriatic lesion, the following alterations have been observed in the stem cell population:

i. The number of basal cells is increased relative to the surface area of the epidermis (5).

ii. The number of stem cells is increased. In normal epidermis, 32% of DNA synthesizing cells are located above the basal layer. In psoriasis, 49% are located above the basal layer (6).

iii. The thymidine labeling index in psoriatic epidermis is six times greater than that of normal epidermis (1).

iv. The duration of the cell cycle is reduced from over 400 hours to about 40 hours (7).

Halprin and Ohkawara (8) have shown that there are no qualitative differences between the enzymes of carbohydrate metabolism in normal and psoriatic epidermis. Many enzymes are moderately elevated in psoriatic epidermis; however, enzymes that catalyze the formation of the pentose ribose-5-P are greatly increased. Ribose-5-P is necessary for nucleic acid synthesis which is greatly increased in psoriatic epidermis. The increased rate of cell division and reduced duration of cell cycle in psoriasis appear to account for the metabolic activity of psoriatic epidermis. The primary disturbance in this disease has not been identified; however, factors controlling the rate of cell division have become a prime target for the study of the pathogenesis of psoriasis.

In 1962 Bullough (9) applied the term "chalone" to a substance that inhibited the rate of cell division of the tissue in which it was manufactured. The concept has been developed by three groups: the London group: Bullough and Laurence (9, 10, 11, 12); the Oslo group: Iversen, Elgjo, and Hennings (13, 14, 15, 16); and the Ann Arbor group: Voorhees, Duell, and Marrs (17, 18, 19). The development of the chalone argument is presented elsewhere in this volume by Elgjo. The important features of the concept are that an epidermal product prevents stem cells from entering mitosis. The experiments of Bullough utilizing surviving segments of mouse ear skin were validated by Marrs and Voorhees who found that an aqueous extract of heat separated epidermis from newborn rats reduced the number of mitoses in surviving mouse ear skin (17, 18).

The abnormal response of psoriatic epidermis to injury is characteristic of that disease. The phenomenon, first described by Koebner (20), has been investigated by means of controlled injury to the epidermis and/or dermis. Suction sufficient to disrupt small blood vessels with resultant petechiae is not followed by the Koebner phenomenon (21). Similarly, incision of the dermis parallel to the skin surface does not result in the Koebner phenomenon. Injury to the epidermis by a number of modalities, including scraping, adhesive cellophane tape stripping or caustic chemicals results in the Koebner phenomenon (21, 22). Injury to normal epidermis is followed by increased DNA synthesis and proliferative activity. In contrast to the normal epidermis in which this response to injury diminishes with healing, the psoriatic epidermis sometimes maintains its high proliferative rate after the injury is healed. A rea-

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sonable model of psoriasis is one in which control of cell division is defective.

The altered cell cycle and transit time of keratinocytes in psoriasis might reflect either a reduced responsiveness to an intrinsic inhibitor or a reduction in the amount of the inhibitor synthesized in the epidermis. A second possibility is that psoriasis represents a stimulated state—that is, a stimulation of stem cell division.

If normal inhibition is lacking in psoriasis, then keratinocytes obtained from normal epidermis should behave similarly to psoriatic keratinocytes when cultivated and it would be predicted that the cell cycle would be constricted in both normal and psoriatic keratinocytes in culture. Fundamental to this argument is that an inhibitor of mitosis is elaborated by differentiated keratinocytes which would be present in reduced numbers in cultures of keratinocytes. The following questions can be posed:

i. does the cell cycle of keratinocytes in psoriatic lesions represent a stimulated or derepressed state?

ii. can cultivated mammalian keratinocytes be used to study the chalone phenomenon?

In this report, we will describe the characteristics of cultivated human and guinea pig keratinocytes. Cultivated keratinocytes form desmosomes *in vitro* and synthesize fibrous material with the ultrastructural appearance of keratin. Evidence will be presented that the keratinocytes cultivated from involved and uninvolved human psoriatic skin have similar mitotic behaviour *in vitro* and that the cell cycle *in vitro* is considerably shorter than that of the normal basal cell *in vivo*. Extracts of rat epidermis that inhibit mitosis in the surviving ear assay will be shown to inhibit mitotic activity of cultivated keratinocytes. Aqueous extracts of liver which are not inhibitory in the surviving mouse ear assay inhibit mitosis of cultivated keratinocytes.

MATERIALS AND METHODS

Keratinocytes were obtained from guinea pig ears or human skin and cultivated either as explants or monolayers. Tissue was obtained either as a 0.1 mm thick layer with a Castroviejo keratotome or with a biopsy punch. For monolayer cultures, the epidermis was removed from the dermis after incubation with trypsin 0.25% (Difco 1:250) at 37° for 20 min in Tyrode solution. The epidermis was then teased with needles and the resulting cell suspension was injected into Cruickshank chambers. The suspending medium was diploid growth medium containing 10% fetal calf serum, penicillin 100 units/ml, streptomycin 100 μg/ml, and amphotericin B 2.5 μg/ml. The chambers were inverted at 37° for 24 hours and then placed with the cover slip on top. The unattached cells were then flushed out of the chamber. The technique has been described by Cruickshank (23) and by Klaus (24).

Colcemid was used to arrest mitosis usually in a concentration of 0.050 μg/ml. The volume of the Cruickshank chamber is 0.2 ml. Chalone was obtained from John Voorhees of the University of Michigan and con-

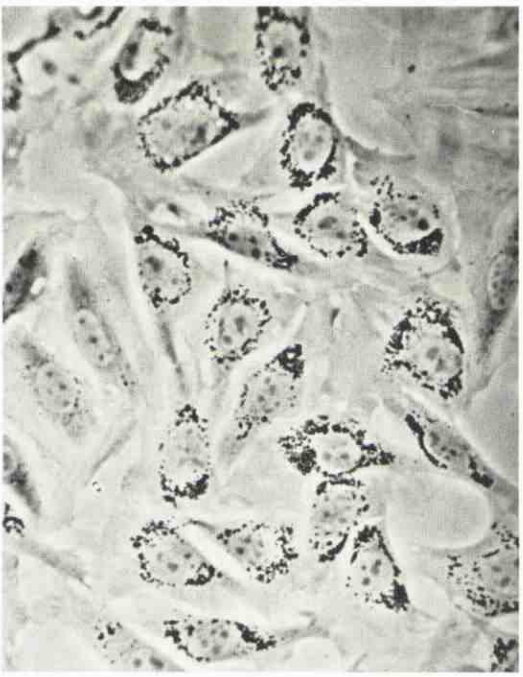

FIG. 1. Keratinocytes obtained from guinea pig ear. These cells have been cultivated in Cruickshank chambers for 5 days. The cells are polygonal and have prominent nuclei and nucleoli. Occasional points of attachment between keratinocytes can be seen. Phase microscopy × 300.

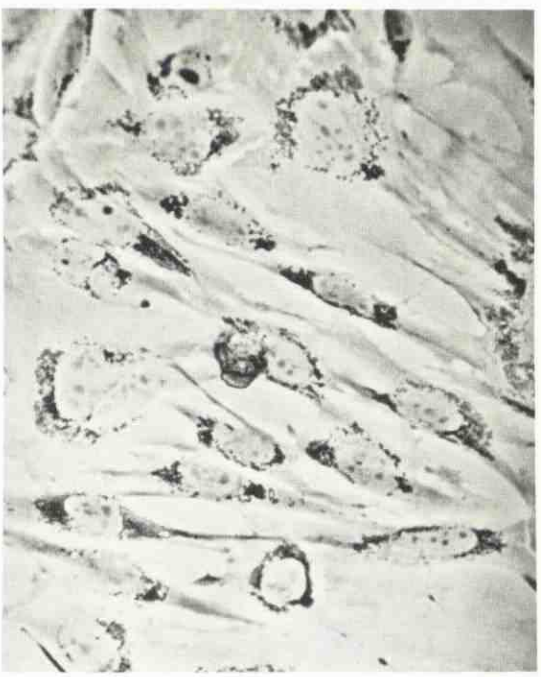

FIG. 2. Keratinocytes obtained from same animal shown in Figure 1. The cells are somewhat more elongated than those shown in Figure 1, and resemble fibroblasts. Electron microscopy shows that these cells contain desmosomes and tonofilaments. Phase microscopy × 300.

tained 0.56 mg protein/mg dry weight. The preparation was a lyophylized aqueous extract from heat separated newborn rat epidermis that had been tested by Voorhees in the surviving mouse ear fragment assay (17). Keratinocytes were also cultivated as explants in plasma clots and as monolayers in Falcon flasks that had been gassed with 10% CO_2 in air. Fibroblasts were cultivated in Falcon flasks. A punch biopsy was trimmed of fat and epidermis and diced with scissors in a few drops of diploid growth medium. The suspension was transferred to Falcon flasks which were gassed with 10% CO_2 in air. Light microscopic observations were made with a Nikon inverted microscope with phase optics. Electron micrographs were made by Dr. Gisela Moellmann of this department. Cells were fixed at 30° in 3% glutaraldehyde and then in 1% osmium tetroxide. They were stained with uranyl acetate and lead citrate. The cultures were embedded in epon by the technique of Branson (25) and then removed from the glass substrate. The cells were sectioned in a plane parallel to the substrate. Cells to be examined for mitoses were fixed and stained as follows. A solution of ethanol, and

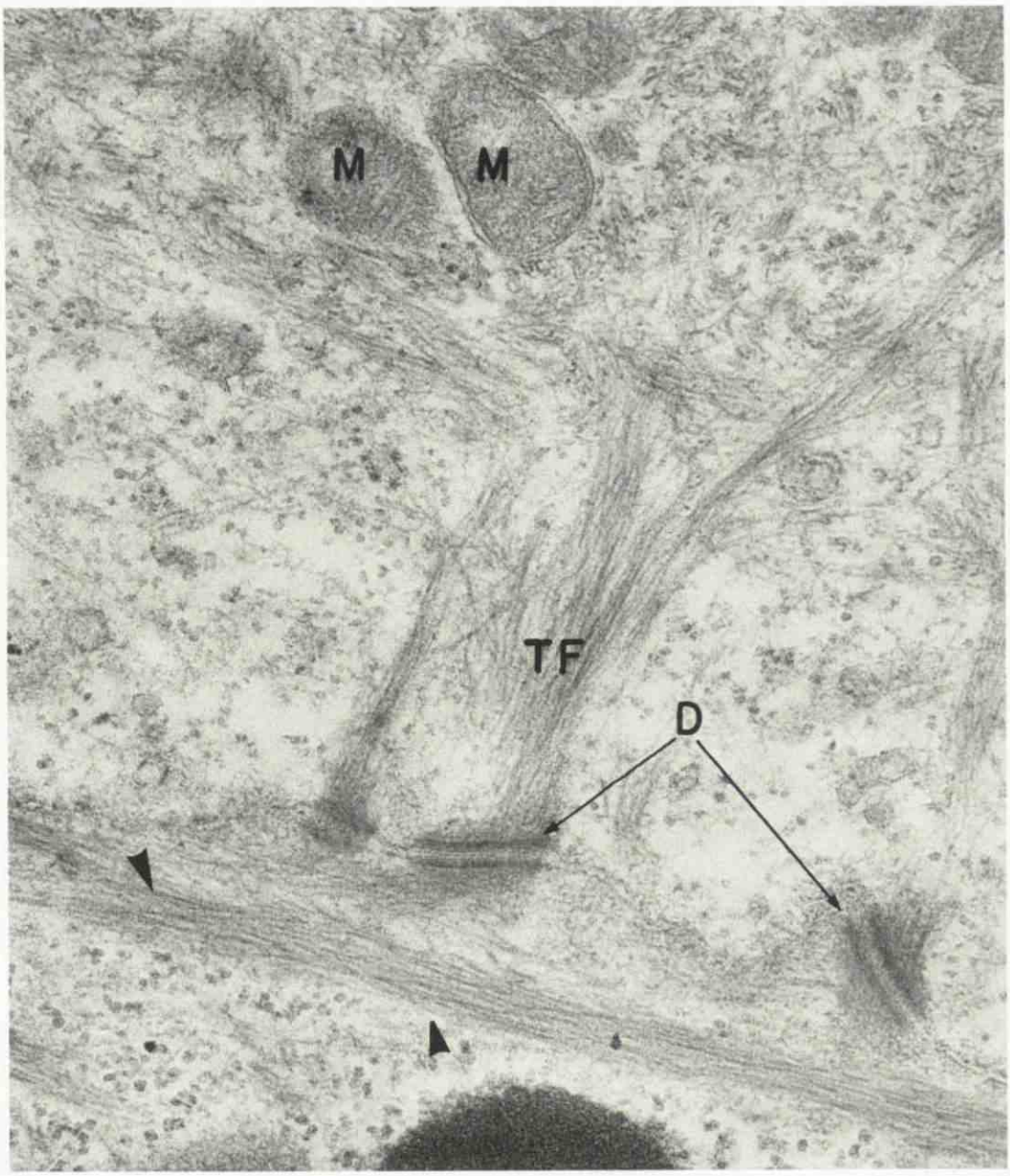

FIG. 3. Electronmicrograph of keratinocytes cultivated from guinea pig epidermis in diploid growth medium in a Cruickshank chamber. Desmosomes (D) are well developed and there are bundles of tonofilaments (TF) streaming toward them. Wisps of filamentous material, presumably keratin, are dispersed throughout the cell. In addition, an orderly array of microfilaments (arrows) is sometimes seen beneath and parallel to the plasma membrane. Mitochondria (M) are present. × 58,300.

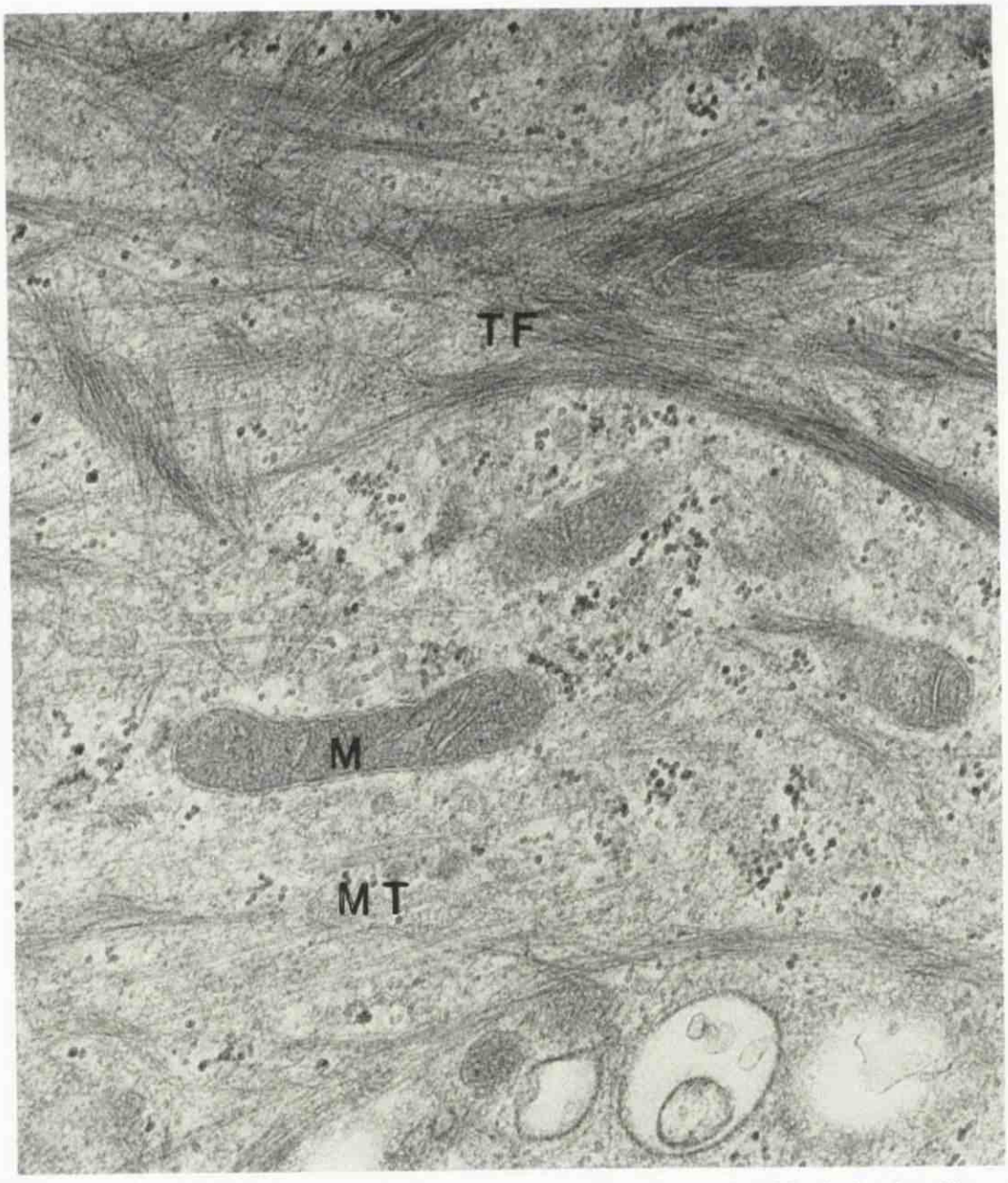

FIG. 4. Electronmicrograph of keratinocyte from guinea pig cultivated in a Cruickshank chamber. There are abundant tonofilaments (TF) and mitochondria (M). Microtubules (MT) are also abundant in these keratinocytes. × 47,700.

acetic acid (3:1) was added to the cultivated cells. The cells were treated twice with the fixative for 10 minutes and air dried. Cells were stained with Wright-Giemsa.

RESULTS

Cultivated keratinocytes have a characteristic appearance by phase contrast microscopy. They are flattened, polygonal and form attachments with neighboring cells. They have prominent nucleoli (Fig. 1). After variable times *in vitro* usually about two weeks, the keratinocytes tend to become more bipolar and less polygonal (Fig. 2). Positive identification of keratinocytes can be made by electron microscopy which shows desmosomes and tonofilaments (Fig. 3). Wisps of keratin filaments, microtubules, and mitochondria are also present in the cultivated cells (Fig. 4). The cultures of guinea pig and human cells contain variable numbers of melanocytes which donate pigment granules to the keratinocytes as has been described (24, 26, 27) (Fig. 5).

The number of keratinocytes moving through

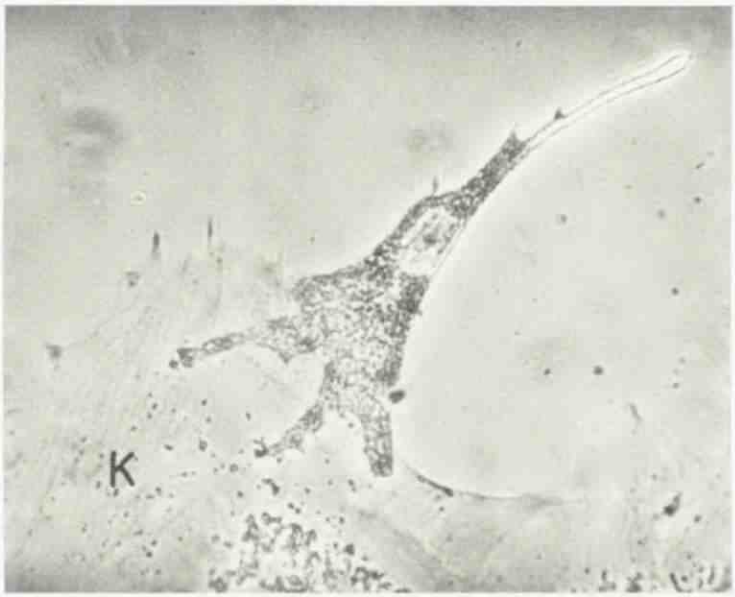

FIG. 5. Interaction between a keratinocyte (K) and melanocyte of guinea pig. The cells were cultivated in a Cruickshank chamber. Melanocytes vary in number; there are usually more melanocytes in cultures obtained from young animals. Phase microscopy × 600.

TABLE I

Relation of the concentration of colcemid to the numbers of mitotic figures observed in guinea pig keratinocytes cultivated in Cruickshank chambers.

Colcemid (μg/ml)	Exposure to colcemid (hours)	Age of culture (days)	Mitoses/1000 cells
6	17	3	30, 20, 28
0.2	17	13	170, 160
0.1	16	9	182, 186
0.05	16	5	260
0.05	16	15	272
0.05	16	12	314
0.05	19	26	408
0.05	19	20	430
0.025	16	5	394
0.025	16	5	424
0.025	16	14	442
0.025	19	20	408
0.025	19	26	430

TABLE II

Reproducibility of mitotic counts in cultivated guinea pig keratinocytes following exposure to colcemid.

Colcemid (μg/ml)	Exposure to colcemid (hours)	Age of culture (days)	Mitoses/1000 cells
0.05	0	8	34
0.05	0	8	38
0.05	5	8	124
0.05	5	8	126

G_2 to M was determined by exposing the cultures to colcemid. The concentration of colcemid is critical; high concentrations of colcemid are toxic and prevent cells from entering M. Based on the data in Table I, a concentration of 0.05 μg/ml was employed in the following experiments. The reproducibility of this technique is indicated in Table II. Scoring of mitosis is much easier with cultivated cells in monolayers than with tissue slices. Mitotic figures are easily recognized. See Figure 6.

Addition of epidermal extracts 250 μg/ml to Cruickshank chambers affected significantly the number of keratinocytes trapped in M by colcemid (Table III). This effect was independent of the duration of cultivation. The degree of inhibition of mitosis was related to the amount of chalone added to the chambers (Table IV). The inhibition of keratinocyte entry into M could also be effected by extracts of liver (Table V).

Keratinocytes and fibroblasts cultivated from involved and uninvolved areas of psoriatic patients showed similar mitotic activity (Table VI).

DISCUSSION

The control of compensatory growth after tissue damage is an important biological puzzle, the elucidation of which offers an increased comprehension of tissue growth that ignors such controls. Psoriatic epidermis is not malignant; however; psoriatic keratinocytes either are not responsive to the normal inhibitor of cell division, lack the inhibitor, or are stimulated by some means to divide. Observations from several labo-

ratories (12, 13, 17) have established the presence in epidermis of an easily extractable material that prevents keratinocytes from entering M. The experiments of Baden and Sviokla (28) are consistent with the site of inhibition to be in G_2. It is possible that such a chemical inhibitor controls mitotic activity in normal epidermis and that reduced levels of such an inhibitor are responsible for the accelerated proliferation of keratinocytes in diseases such as psoriasis, epidermolytic hyperkeratosis and lamellar ichthyosis in all of which the keratinocyte transit time, and presumably cell cycle, are abbreviated.

The synergism of epinephrine in the inhibition of keratinocyte mitosis by epidermal extracts (11) suggests the possibility that adenosine (3',5'-monophosphate (c-AMP) may be involved in the control of cell division. Adenyl cyclase, the enzyme that catalyses the synthesis of c-AMP from ATP is stimulated in various tissues by many effectors, including epinephrine. Recently, investigators in Oslo have demonstrated an increase in c-AMP synthesis in hamster epidermis in the presence of micromolar concentrations of epinephrine (29).

It is tempting to construct a model of keratinocyte division in which cyclic-AMP is an important regulator. Supporting this hypothesis is that c-AMP inhibits growth of cultivated cells *in vitro*. Less encouraging is the observation that non-malignant cells are less sensitive to inhibition by c-AMP (30).

TABLE III

Reduction of mitoses in guinea pig keratinocytes by the addition of epidermal extracts. Keratinocytes were cultivated in Cruickshank chambers for 14 days and exposed to colcemid or epidermal extract and colcemid for 20 hours.

Addition (μg/ml)	Mitoses/1000 cells
Colcemid 0.05 μg	280, 292
Colcemid 0.05 μg + epidermal extract 250 μg/ml	118, 128

TABLE IV

Inhibition of mitosis by epidermal extracts added to Cruickshank chambers containing guinea pig keratinocytes. Cells have been grown for 11–13 days in diploid growth medium and exposed to colcemid or colcemid and epidermal extract for 17 hours.

Epidermal extract (μg/ml)	Mitoses/1000 cells
0	240, 266
125	164, 158
250	106
500	74, 94
1,000	80

TABLE V

Inhibition of mitosis by extracts of liver and epidermis

Keratinocytes were exposed to colcemid 0.05 μg/ml and an addition, for 16 hours.

Age of culture (days)	Mitoses/1000 cells		
	Addition (μg/ml)		
	0	Liver extract 250	Epidermal extract 250
11	174, 174	66	60
20	186	74, 70	54

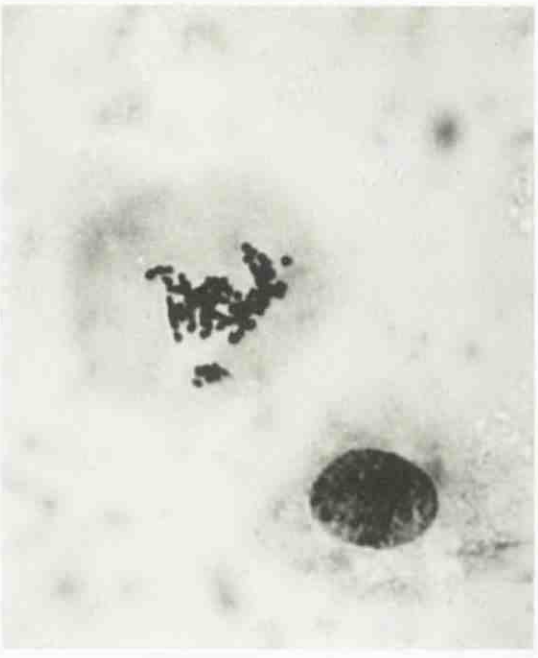

FIG. 6. Mitosis of guinea pig keratinocytes arrested with colcemid 0.05 μg/ml. The cells have been stained with Wright-Giesma. × 600.

TABLE VI

Cells were exposed to colcemid for 6 hours. Keratinocytes from involved and uninvolved areas had similar mitotic rates *in vitro*. Fibroblasts showed similar rates of mitosis.

	Mitoses/1000 cells from psoriatic skin	
	Uninvolved	Involved
Fibroblasts	36, 51	32, 47
	53, 40	43, 24
	34, 52	24, 40
	36	50, 40
Keratinocytes	50	60
	48	44
	56	62
		68

CONCLUSIONS

1. Aqueous extracts of epidermis inhibit mitotic activity of cultivated keratinocytes.

2. The degree of inhibition of mitosis by epidermal extracts is dose related.

3. In the assay of chalone activity employing cultivated keratinocytes, specificity is lacking; extracts of liver are as inhibitory as extracts of epidermis.

4. Fibroblasts and keratinocytes cultivated from involved and uninvolved skin of psoriatic patients have similar mitotic indices.

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