0022-202X/83/8101S-0033s$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 81:33s–40s, 1983

Vol. 81, No. 1 Supplement
Printed in U.S.A.

Calcium-Regulated Differentiation of Normal Human Epidermal Keratinocytes in Chemically Defined Clonal Culture and Serum-Free Serial Culture

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An improved serum-free culture system has been developed for normal human epidermal keratinocytes (HK). Short-term clonal growth and differentiation studies are routinely performed in a defined medium consisting of optimized nutrient medium MCDB 153 supplemented with epidermal growth factor, insulin, hydrocortisone, ethanolamine, and phosphoethanolamine. A small amount of whole bovine pituitary extract (wBPE) is added for initiation of primary cultures, for frozen storage, and for serial culture. The need for feeder layers, conditioned medium, serum, and specialized culture surfaces has been eliminated entirely. With an optimal level of calcium ion (0.3 m*M*), colony-forming efficiency is about 30 percent and cellular multiplication rate is 0.96 doublings per day in the defined medium. A high-calcium concentration (1.0 m*M*) induces stratification and terminal differentiation, which can be quantified by counting cornified envelopes that are resistant to boiling in sodium dodecyl sulfate plus dithiothreitol. Under optimal conditions with wBPE present, cellular senescence occurs after about 40 population doublings. Scanning electron microscopy (SEM) has verified the occurrence of stratification during differentiation in the defined medium with high calcium. High-voltage electron microscopy (HVEM) after detergent extraction of human epidermal keratinocyte (HK) colonies grown in the defined medium with low and high calcium has revealed specific changes in the intermediate filament network and keratohyalin granules corresponding to changes in cellular differentiation. Indirect immunofluorescence studies have verified that the intermediate filament network observed with HVEM is composed of keratin proteins.

Reports from this laboratory have described development of an optimized basal nutrient medium for normal human epidermal keratinocytes (HK) [1] and clonal growth of second-culture HK cells in a hormone- and growth factor-supplemented defined medium [2], and improved methods for isolation, serum-free stock culture, and frozen storage of HK cells [3].

This report describes quantitative and qualitative characteristics of (1) growth and differentiation of third-culture HK cells in defined medium at clonal cell densities; (2) serial culture of HK cells in medium containing 0.1 m*M* Ca^{2+}, chemically defined supplements, and whole bovine pituitary extract (wBPE); and (3) changes in surface morphology and intermediate filament organization corresponding to differentiation in vitro as mediated by calcium ion concentration in defined medium.

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Abbreviations:
CFE: colony-forming efficiency
EGF: epidermal growth factor
HK: human epidermal keratinocyte
HVEM: high-voltage electron microscopy
PD: population doublings
SEM: scanning electron microscopy
wBPE: whole bovine pituitary extract

MATERIALS AND METHODS

Culture Procedures

Methods for isolation, primary culture, and frozen storage of human keratinocytes have been described in detail elsewhere [3]. Preparation of wBPE has been previously described [2]. MCDB 153 is MCDB 152 [2] with $FeSO_4$ increased to 5.0×10^{-6} *M* and $ZnSO_4$ reduced to 5.0×10^{-7} *M*.

The defined medium for clonal growth assays consisted of MCDB 153 supplemented with 5 ng/ml epidermal growth factor (EGF), 5 μg/ml insulin, 1.4 μ*M* (0.5 μg/ml) hydrocortisone, 0.1 m*M* ethanolamine, and 0.1 m*M* phosphoethanolamine. MCDB 153 contains 0.03 m*M* $CaCl_2$. For many experiments, the total $CaCl_2$ concentration was increased to 0.1, 0.3, or 1.0 m*M*, as described in the text. The stock culture medium for primary cultures and for serial cultures was identical to the clonal growth medium except that it contained 10 ng/ml EGF and 0.1 m*M* Ca^{2+} and was supplemented with wBPE at a protein concentration (Lowry) of 70 μg/ml.

As they were needed for serial culture or clonal growth experiments, ampules of cells, frozen after primary culture, were thawed at 37°C in 70% ethanol, and the cells were inoculated at a density of 500 to 3000 cells per square centimeter into flasks of stock culture medium that had been equilibrated in the cell-culture incubator for at least 30 minutes. After 24 hours, the medium was removed, the cultures were washed with Solution A (a Ca^{2+}- and Mg^{2+}-free HEPE's-buffered saline solution), and fresh medium was added. For best results, it was important to harvest the stock cultures while they were still actively multiplying. They were generally ready to use 3 to 7 days after inoculation.

For long-term serial culture experiments, cells were inoculated at a fixed density of 400 cells per square centimeter in stock culture medium with penicillin-streptomycin-fungizone (Gibco no. 600-5245). The cultures were harvested, counted, and subcultured when they were judged to be more than half confluent or after 7 days, whichever came first. At each subculture, parallel cultures were initiated on 60-mm petri dishes with grids (Costar no. 3160). After 4 to 6 days, the grid dishes were fixed with 2% glutaraldehyde, stained with 0.1% crystal violet, and scored for colonies formed.

For clonal growth assays, an inoculum of 500 second-culture HK cells (25 cells/cm^2) was added to each 60-mm petri dish containing 5.0 ml of clonal growth medium. The dishes containing the medium were equilibrated in the cell-culture incubator (37°C, 5% CO_2, saturated humidity) for at least 30 minutes prior to inoculation. Control dishes containing clonal growth medium plus wBPE (70 μg/ml protein) were included in all clonal growth experiments. The incubation period was 10 to 11 days, without medium change. At the end of the assay period, some of the dishes were fixed, stained, and scored for colonies formed per dish, as in the serial culturing experiments. Identical dishes were washed with Solution A, and the cells they contained were harvested by trypsinization followed by scraping with a rubber policeman. These cell suspensions were pelleted by centrifugation at 250g for 5 minutes, resuspended in small volumes of medium, and a small sample of each suspension was counted. The remaining suspensions were centrifuged to pellets for a second time, resuspended in 5-ml aliquots of a solution of 1% sodium dodecyl sulfate (SDS) plus 20 m*M* dithiothreitol (DTT) in Solution A, and incubated in a 90°C water bath for 10 minutes. The SDS-DTT insoluble cornified envelopes were then centrifuged to a pellet, resuspended in 0.5 ml of Solution A, and counted.

Data points for serial culture and clonal growth experiments are the average of duplicate or triplicate HK cultures from two separate experiments performed with HK cell strains derived from different tissue donors.

Definitions and Quantitative Formulas

A *colony* is defined as a group of four or more contiguous cells that are judged by their appearance to have arisen from a single cell.

Colony-forming efficiency (CFE), expressed as a percentage, is defined as follows:

$$\text{CFE} = \frac{\text{colonies formed at end of growth period}}{\text{cells inoculated at start of growth period}} \times 100$$

Average population doublings (PD) is expressed as follows:

$$\text{PD} = \log_2 \left(\frac{\text{total cells harvested} - \text{colony-incompetent cells}}{\text{total colonies formed}} \right)$$

Growth rate is expressed as average population doublings per day. *Serial culture number* refers to the number of times a cell population has been grown sequentially in culture, including the primary culture. *Subculture* is the transfer of a cell population from one culture vessel to another. Thus the transfer of a primary culture (P) to another culture vessel generates the second serial culture, which is the result of, and is synonymous with, the first subculture.

Electron Microscopy

Scanning electron microscopy (SEM) specimen preparation followed the methods of Wolosewick and Porter [4] with slight modifications. Samples for SEM were coated with gold-palladium.

High-voltage electron microscopy (HVEM) specimens were grown on Formvar-coated gold grids that had been carbon-coated, glow-discharged, and sterilized with UV irradiation. The cells were detergent-extracted by the method of Schliwa and Van Blerkom [5] with 0.1% triton X-100 extraction, 1% glutaraldehyde fixation, 0.5% OsO_4 postfixation, ethanol dehydration, and critical-point drying.

RESULTS

Clonal Growth of Third-Culture HK Cells in Defined Medium

Colony-forming efficiency (CFE) of HK cells does not vary greatly with concentration of Ca^{2+} ion (Fig. 1). In defined medium, CFE ranges from a minimum of 26.1 percent at 1.0 m*M* Ca^{2+} to a maximum of 29.3 percent at 0.3 m*M* Ca^{2+}. CFE in control dishes containing defined medium plus wBPE ranges from a minimum of 28.4 percent at 0.03 m*M* Ca^{2+} to a maximum of 36.2 percent at 0.3 m*M* Ca^{2+}.

Total number of HK cells resulting from clonal growth at Ca^{2+} concentrations between 0.03 and 1.0 m*M* is shown in Fig. 2. In defined medium, HK cell number increases sharply between 0.03 and 0.1 m*M* Ca^{2+} and continues to rise more slowly to a maximum at 0.3 m*M* Ca^{2+}. However, at 1.0 m*M* Ca^{2+} in the defined medium, there is a dramatic reduction in number of cells generated during the same assay period. Control dishes with wBPE exhibit increases in cell number similar to dishes with defined medium between 0.03 and 0.1 m*M* Ca^{2+} and somewhat greater than in defined medium between 0.1 and 0.3 m*M* Ca^{2+}. Total cell number in cultures with wBPE also decreases between 0.3 and 1.0 m*M* Ca^{2+}, but not as dramatically as in the defined medium.

Measurement of percent cells with cornified envelopes (Fig. 3) demonstrates that even in cultures grown at low (0.03 m*M*) Ca^{2+}, some cornified envelopes are produced. Cornified envelopes represent a significant percentage (7.3–10%) of cells in unstratified cultures grown at 0.03 m*M* Ca^{2+}, because total cell number is reduced at this Ca^{2+} concentration both in defined medium and in cultures containing wBPE (see Fig. 2). Percent cells with cornified envelopes is less in cultures with 0.1 to 0.3 m*M* Ca^{2+} because of increases in total cell number at these calcium concentrations (Fig. 2). Cells with cornified envelopes constitute less than 4 percent of all HK cells grown in 0.1 and 0.3 m*M* Ca^{2+}. Clonal cultures grown in defined medium containing 1.0 m*M* Ca^{2+} show a significant increase in percent

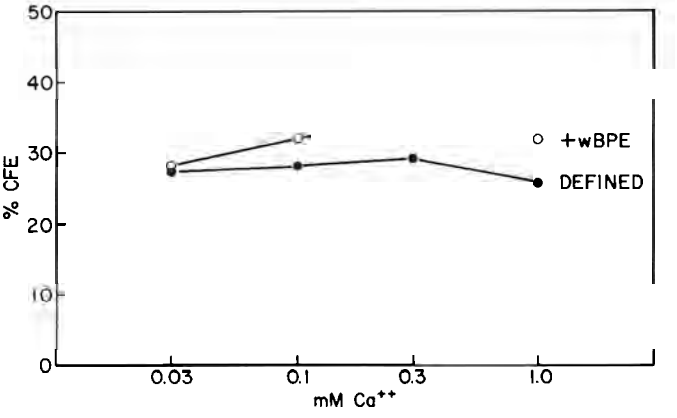


FIG 1. Effect of extracellular calcium ion concentration on colony-forming efficiency in HK clonal cultures. ● = defined medium; ○ = defined medium plus wBPE (70 μg/ml protein).

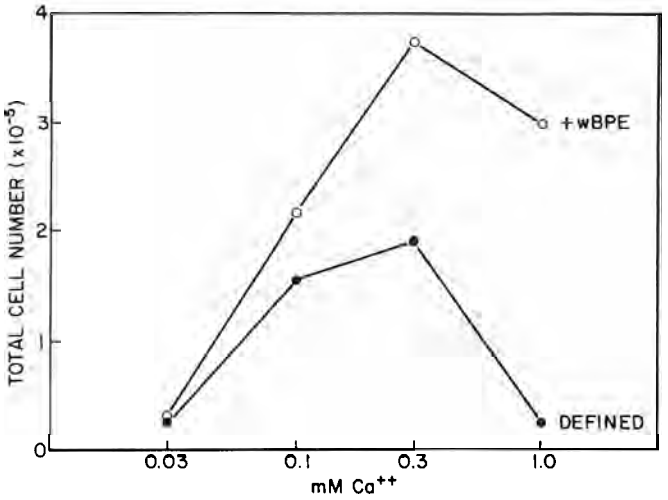


FIG 2. Effect of extracellular calcium ion concentration on total cell number produced in HK clonal cultures with a 500-cell inoculum. ● = defined medium; ○ = defined medium plus wBPE (70 μg/ml protein).

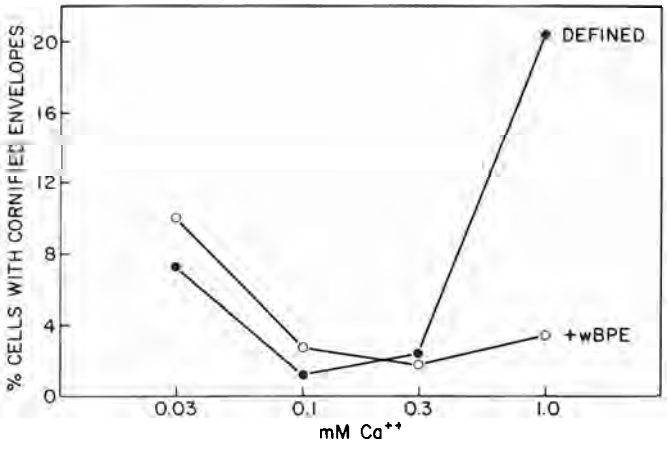


FIG 3. Effect of extracellular calcium ion concentration on percent cells with cornified envelopes in HK clonal cultures. ● = defined medium; ○ = defined medium plus wBPE (70 μg/ml protein).

cornified envelopes due to full stratification and terminal differentiation. Cultures containing wBPE show only a slight change in percent cornified envelopes between 0.3 and 1.0 m*M* Ca^{2+}.

Growth rate of clonal cultures of HK cells (Fig. 4) increases between 0.03 and 0.1 m*M* Ca^{2+} and again between 0.1 and 0.3 m*M* Ca^{2+} concentrations. Maximum growth rate for cultures in defined medium is 0.96 population doublings per day, and for cultures containing wBPE, it is 1.06 population doublings per day. Thus, over a 10- to 11-day assay period, maximum proliferation in the defined medium is only about one population doubling less than with wBPE present (Fig. 2). Both growth rate maxima occur at 0.3 m*M* Ca^{2+}. Between 0.3 and 1.0 m*M* Ca^{2+}, growth rate of cultures containing wBPE is essentially unchanged, whereas growth rate in the defined medium with 1.0 m*M* Ca^{2+} drops to 0.72 doublings per day.

Serial Culture of HK Cells

Under the serial culture conditions described in *Materials and Methods*, CFE of HK cells increases from about 1 to 2 percent in primary culture [3] to a maximum of 33.9 percent in third serial culture cells (Fig. 5). Between third and seventh serial cultures, CFE steadily declines, until only 1.4 percent of the cells retain enough replicative ability to form a four-cell colony in 7 days.

Growth rate of HK cells decreases each time the cells are subcultured (Fig. 6). The rate declines from 0.95 doublings per

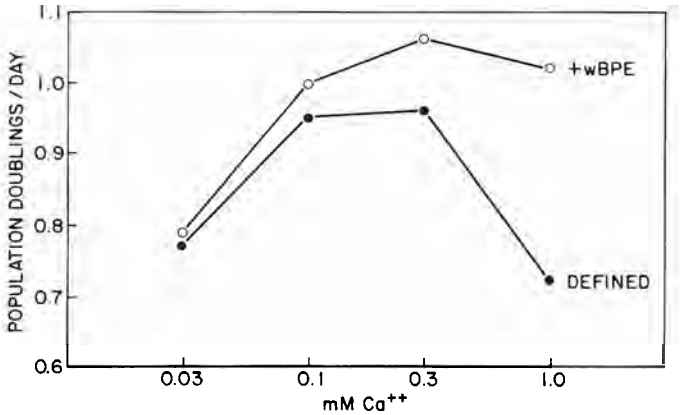


FIG 4. Effect of extracellular calcium ion concentration on growth rate (PD/day) in HK clonal cultures. ● = defined medium; ○ = defined medium plus wBPE (70 μg/ml protein).

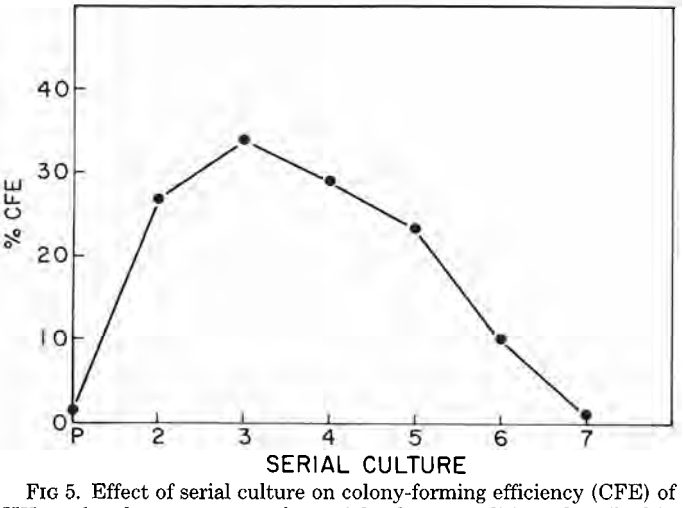


FIG 5. Effect of serial culture on colony-forming efficiency (CFE) of HK stock cultures grown under serial culture conditions described in *Materials and Methods.*

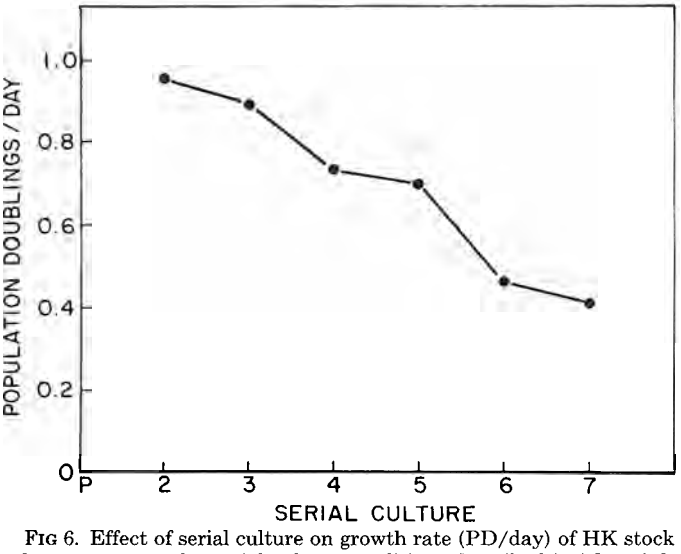


FIG 6. Effect of serial culture on growth rate (PD/day) of HK stock cultures grown under serial culture conditions described in *Materials and Methods.*

day for second serial culture cells to 0.41 doublings per day for seventh serial culture cells. At seventh serial culture, most of each culture was composed of enlarged, flattened cells. Such cultures were considered senescent, and subculturing was discontinued.

Summation of cumulative population doublings from these experiments shows that HK cells grown under these conditions divide 28 to 29 times between the second and seventh serial cultures. We estimate that the cells divide an average of 10 to 12 times in primary culture. Therefore, the in vitro lifespan of HK cells in this culture system is about 40 population doublings.

Scanning Electron Miscroscopy (SEM)

SEM of HK cultures grown in defined medium containing 0.03, 0.1, 0.3, or 1.0 m*M* Ca^{2+} (Fig. 7) illustrates the effects of calcium concentration on stratification of HK cells in vitro. Virtually no stratification is seen at either 0.03 m*M* (Fig. 7*A*) or 0.1 m*M* (Fig. 7*B*). At 0.3 m*M* Ca^{2+} (Fig. 7*C*), local areas of stratification begin to appear within large colonies. Full stratification occurs concurrently with reduced colony size and increased production of cornified envelopes (Fig. 7*D*) in defined medium containing 1.0 m*M* Ca^{2+}.

High-Voltage Electron Microscopy (HVEM)

Triton extraction and subsequent HVEM observation of HK cells grown in defined medium containing 0.03, 0.1, 0.3, or 1.0 m*M* Ca^{2+} concentrations reveals changes in the intermediate filament (IF) organization of HK cells associated with differentiation in vitro.

Cells grown in 0.03 m*M* Ca^{2+} are unstratified, flattened, and

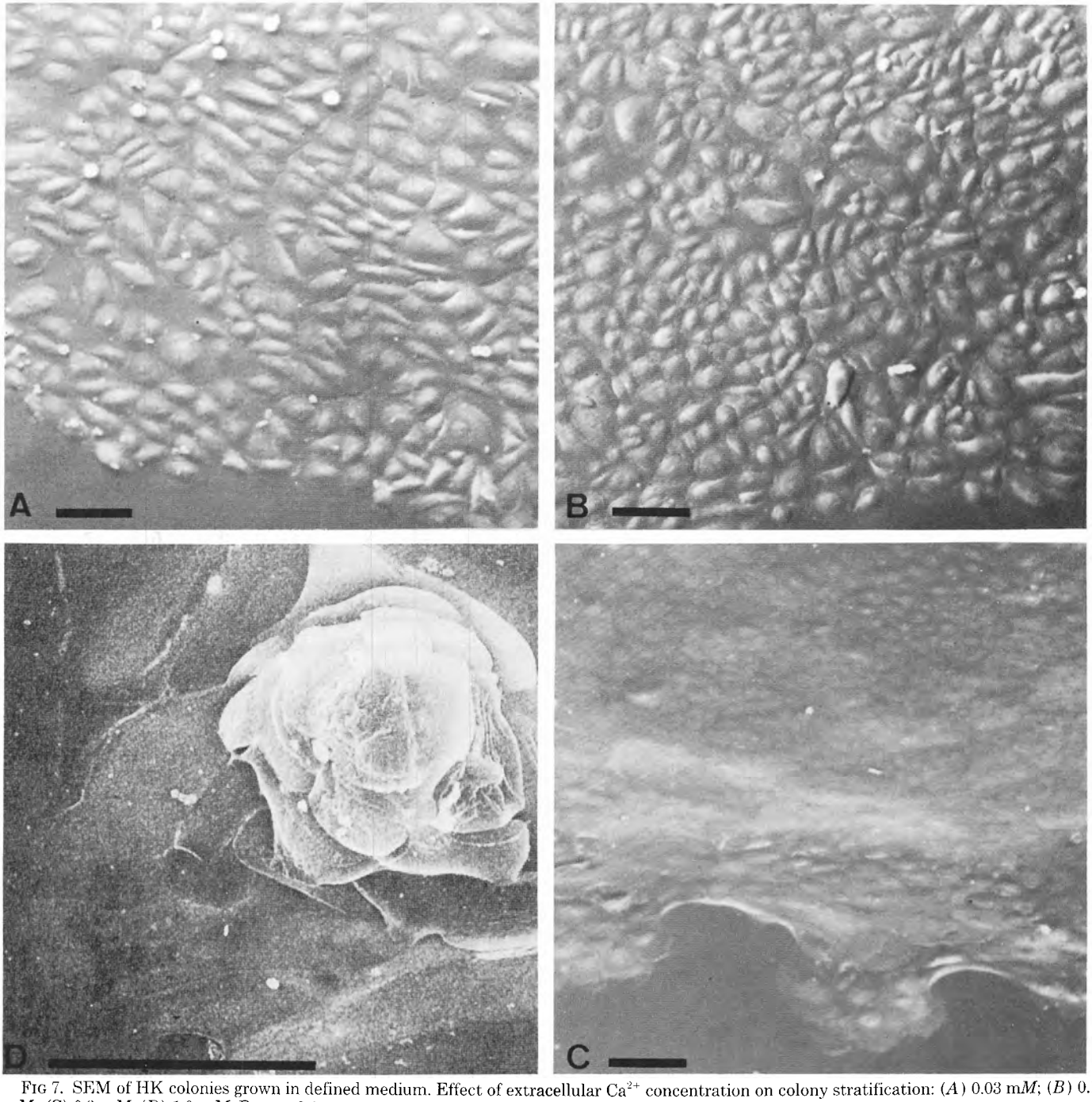


FIG 7. SEM of HK colonies grown in defined medium. Effect of extracellular Ca^{2+} concentration on colony stratification: (*A*) 0.03 m*M*; (*B*) 0.1 m*M*; (*C*) 0.3 m*M*; (*D*) 1.0 m*M*. Bars = 0.1 mm.

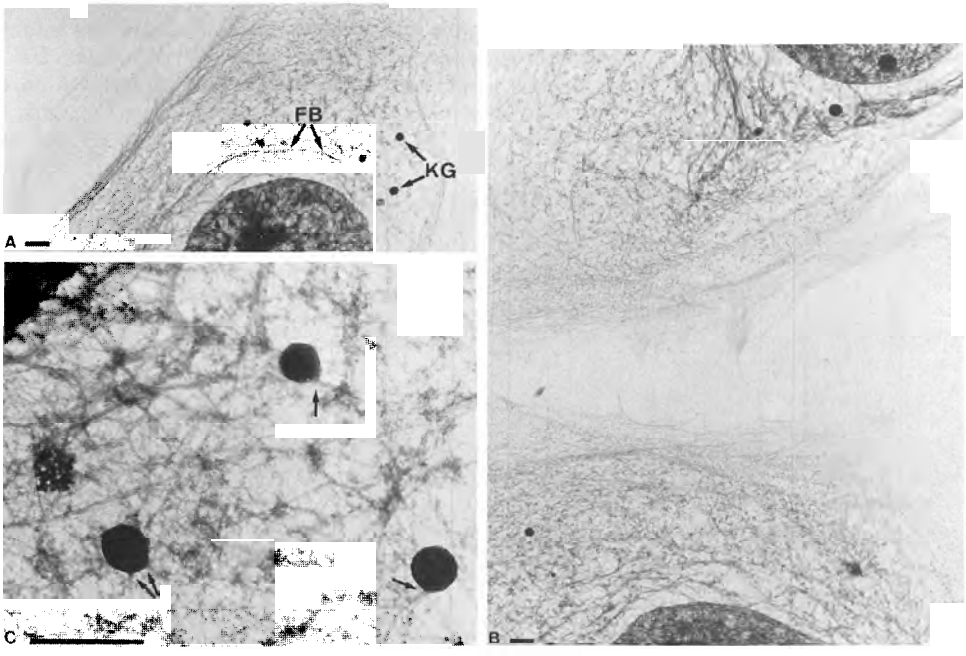


FIG 8. HVEM of triton-extracted HK cells grown in defined medium with 0.03 m*M* Ca^{2+}. *A*, Intermediate filament bundles (FB) and keratohyalin granules (KG) are generally perinuclear in distribution. *B*, Very few intercellular filaments are seen. *C*, Intermediate filaments are joined (*black arrows*) to keratohyalin granules. Bars = 1 μm.

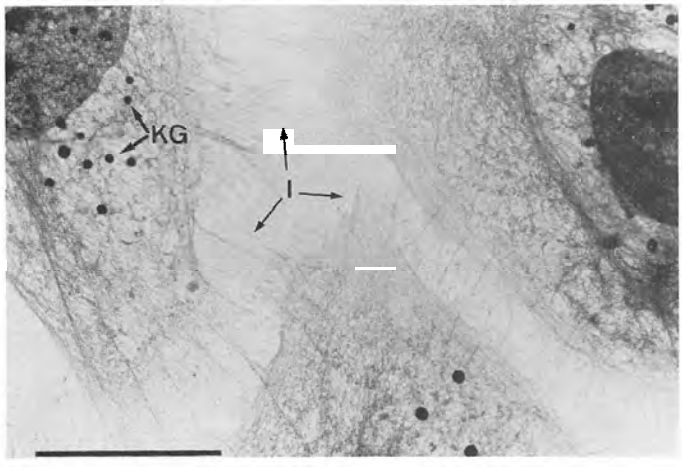


FIG 9. HVEM of triton-extracted HK cells grown in defined medium in 0.1 m*M* Ca^{2+}. Intracellular intermediate filaments (*I*) become very frequent and keratohyalin granules (*KG*) also increase in number. Bar = 10 μm.

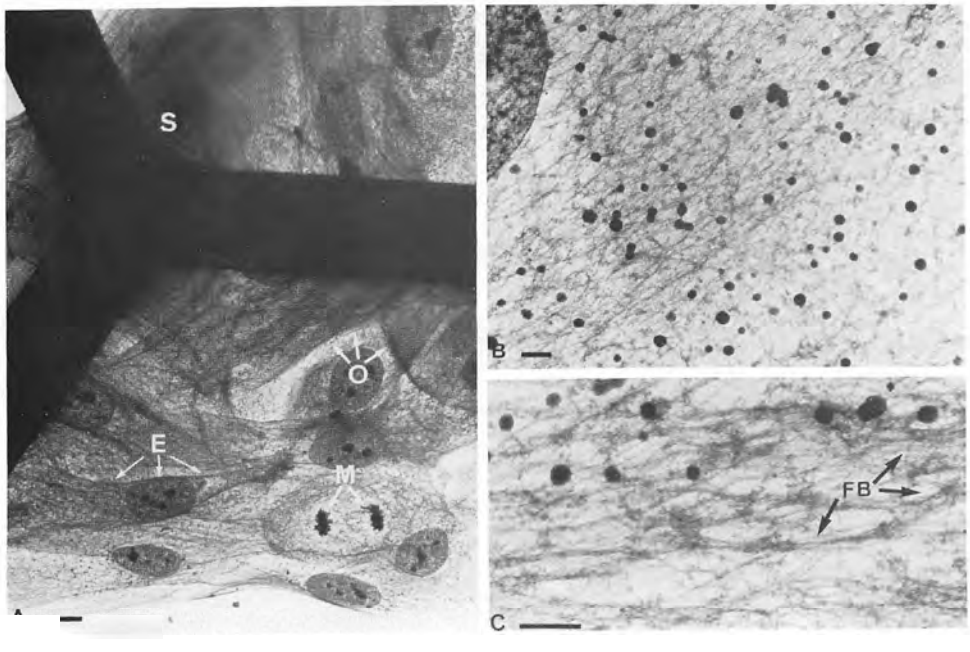
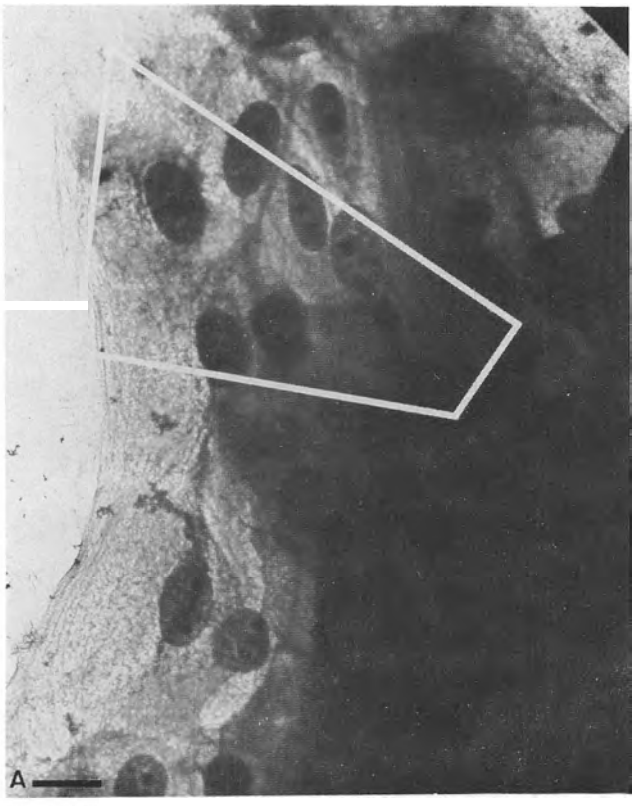


FIG 10. HVEM of triton-extracted HK cells grown in defined medium with 0.3 m*M* Ca^{2+}. *A*, A mitotic figure (*M*) is seen near the edge of a colony of cells. Intermediate filament bundles of some cells show bipolar extension (*E*) and overlap (*O*) as stratification occurs. The partially stratified apex (*S*) of this colony is a dense mat of intermediate filaments. Bar = 10 μm. *B*, Increased numbers of keratohyalin granules are seen. *C*, Increased numbers of intermediate filament bundles also occur. *B*,*C* Bars = 1 μm.

exhibit an organization of IFs and keratohyalin granules that is generally perinuclear (Fig. 8*A*). There are only a very few intercellular IFs that radiate from the cell perpendicular to the nucleus (Fig. 8*B*). Higher magnification demonstrates the contiguity of keratohyalin granules with the IF framework of the cell (Fig. 8*C*). At 0.1 m*M* Ca^{2+}, the cells develop very frequent intercellular IFs, and the number of keratohyalin granules per cell also increases (Fig. 9). As seen in SEM (Fig. 7*C*), stratification accompanies HK growth in 0.3 m*M* Ca^{2+} (Fig. 10*A*). At this Ca^{2+} concentration, the elaboration of IFs and keratohyalin granules (see Fig. 10*B*, *C*) appears to be greater than can be accounted for by the additive effects of stratification alone.



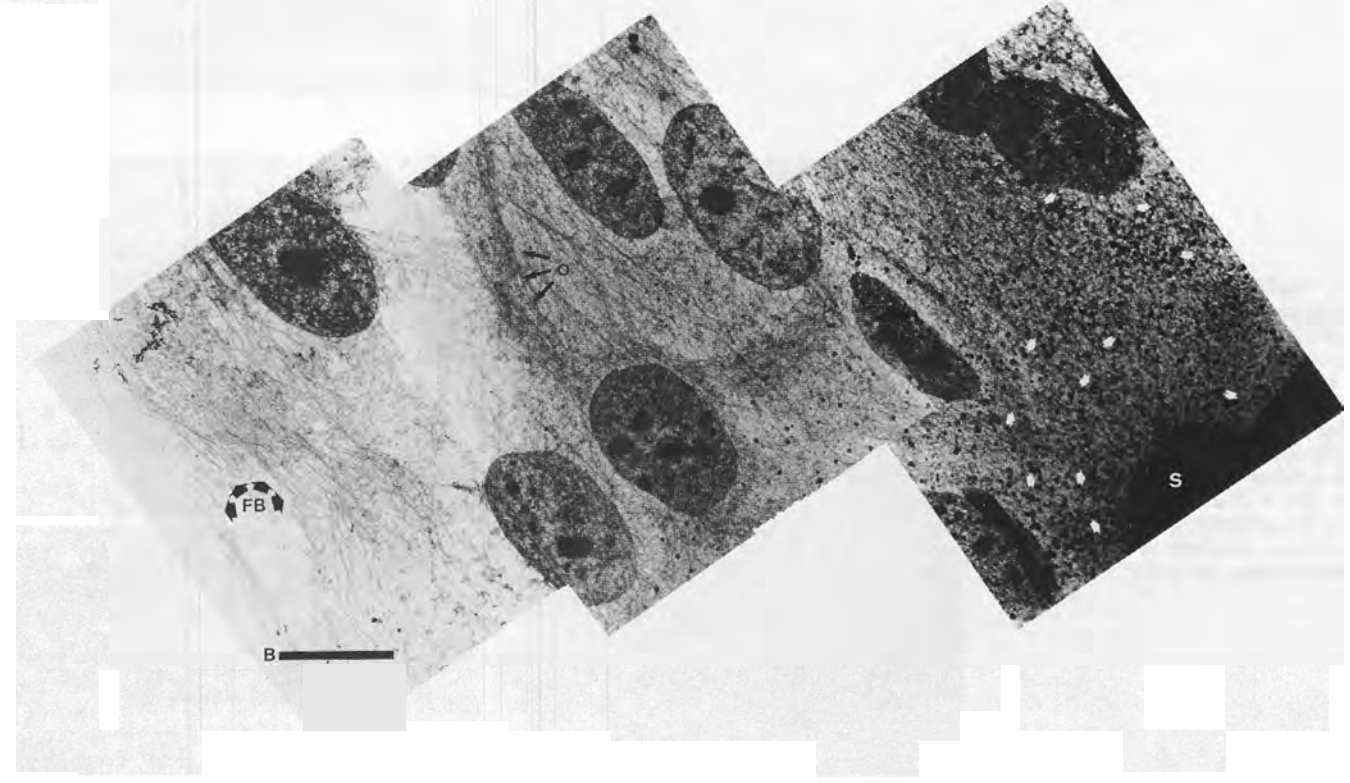


FIG 11. HVEM of a fully stratified HK colony grown in defined medium with 1.0 m*M* Ca^{2+}. *A*, Overlying strata make resolution difficult. Area shown in Fig. 11*B* is outlined in white. *B*, Composite micrograph showing intermediate filament bundles (*FB*) at colony edge, overlapping intermediate filament networks (*O*) of stratified cells, and greatly increased numbers of keratohyalin granules (*white arrows*) adjacent to the fully stratified center of the colony (*S*). Bars = 10 μm.

Terminal differentiation and full stratification of HK cells are observed at 1.0 mM Ca^{2+} (Fig. 11A). The stratified centers of these colonies are thick enough to prevent the penetration of the HVEM beam and therefore appear black. However, a composite micrograph (Fig. 11B) clearly demonstrates that IFs and keratohyalin granules increase in number near the center of this fully stratified colony. This implies that increased production of keratohyalin granules occurs in a suprabasal cellular stratum analogous to epidermal differentiation in vivo.

Indirect Immunofluorescence

Using a rabbit antiserum directed against human epidermal keratin proteins, we have confirmed that the intermediate filament network of detergent-extracted HK cells observed by HVEM is composed of keratin proteins (Fig. 12A). Swiss 3T3 cells were used as negative controls (Fig. 12B).

DISCUSSION

The data presented in this report conclusively confirm that feeder cells, substrate modifications, and serum are not required for clonal growth, serial culture, and differentiation in vitro of normal human epidermal keratinocytes. In this laboratory it has been demonstrated that suboptimal or excessive amounts of essential nutrients, such as amino acids, vitamins, and inorganic ions, can be growth-limiting for HK as well as other mammalian cells in culture [6]. Hence use of a nutrient medium that is qualitatively and quantitatively optimized for HK cells, such as MCDB 153, improves HK growth by increasing amounts of nutrients that are deficient and by reducing amounts of nutrients that are in toxic excess, as well as by supplying nutrients that are missing from conventional media.

Agents such as cholera toxin that elevate cAMP levels have been reported to increase HK proliferation in vitro [7]. Similarly, changes in extracellular calcium ion concentration have been shown both to increase intracellular cAMP levels and to initiate DNA synthesis in normal peripheral lymphocytes and hepatocytes from rats [8]. Our data from clonal assays confirm that the growth rate of HK cells increases with added extracellular Ca^{2+} between concentrations of 0.03 and 0.3 mM and is nearly maximal at 0.1 mM. In defined medium containing 1.0 mM Ca^{2+}, HK growth rate is reduced and terminal differentiation is induced. Conversely, 1.0 mM Ca^{2+} greatly increases the growth in culture of normal human fibroblasts, and 0.1 mM Ca^{2+} is suboptimal for their proliferation [9]. Therefore, 0.1 mM extracellular Ca^{2+} causes the in vitro growth rate of keratinocytes to increase and that of fibroblasts to decrease. The differential regulation of cellular multiplication by extracellular Ca^{2+} is an important factor contributing to selective proliferation of HK and simultaneous suppression of fibroblastic growth.

The presence of cornified envelopes in unstratified clonal cultures grown at 0.03 and 0.1 mM Ca^{2+} shows that HK differentiation is not directly dependent on stratification. Watt and Green [10] have shown that HK cells that have been restricted from stratifying by growth in 0.1 mM Ca^{2+} are still competent to produce involucrin, a protein subunit of cornified envelopes. Rheinwald and Beckett [11] have described the commitment of HK cells to terminal differentiation after deprivation of substrate attachment by suspension in methylcellulose. Our findings that some cornified envelopes appear in unstratified cultures suggests that a subset of unstratified dividing cells commits to terminal differentiation at each subculture. The commitment of these cells to terminal differentiation may result from their deprivation from surface attachment during subculture.

SEM of HK cells grown in defined medium at different Ca^{2+} concentrations demonstrates that stratification of human epidermal cells in vitro is dependent on extracellular calcium ion concentration. Regulation of stratification of epidermal cells by extracellular calcium has been shown by Peehl and Ham [1] for human keratinocytes and by Hennings et al. [12] for mouse keratinocytes. More recently, Jones et al. [13] have demonstrated that formation of desmosomes between mouse epidermal cells can be induced by increasing the extracellular Ca^{2+} concentration. We believe that calcium-dependent formation of desmosomes is an important factor in regulation of epidermal stratification and differentiation. However, our microscopy studies show that calcium regulates stratification and differentiation in a stepwise manner corresponding to increasing amounts of extracellular calcium. Intercellular filaments occur between unstratified cells beginning at 0.1 mM Ca^{2+}. Increased numbers of intermediate filament bundles accompany limited colony stratification at 0.3 mM Ca^{2+}. Levels of filaggrin proteins [14], which have been shown to contribute to the formation of intermediate filament bundles, may be elevated in HK cultures grown in 0.3 mM Ca^{2+}. Terminal differentiation and the loss of proliferative capacity induced by 1.0 mM Ca^{2+} coincides with the production of large numbers of keratohyalin granules and cornified envelopes in fully stratified colonies. Thus we are now in a position to segregate the component processes of human epidermal differentiation and determine how these processes are regulated.

Growth rate and CFE of HK cells both decline between the third and seventh serial cultures. Inability to proliferate and enlargement of most cells, together with a decreased growth rate of remaining proliferative cells, is comparable with the phenomenon of in vitro senescence described by Hayflick [15] for normal human fibroblast cells.

In defined medium containing 1.0 mM Ca^{2+}, terminal differentiation is initiated and cellular proliferation is partially blocked. Malignant transformation of human [11] and mouse [16] epidermal cells allows some of those cells to escape the proliferation block associated with terminal differentiation. Such malignant cells are also immortal in culture. The two cell strains used in this study both formed large proportions of cornified envelopes in 1.0 mM Ca^{2+} in defined medium. In addition, both HK strains exhibited finite lifespans under culture conditions that restricted terminal differentiation. Thus, with regard to cornified envelope production and finite in vitro lifespan, the serially cultivated HK strains used in this study behaved as expected for normal, nontransformed cells.

In summary, a culture system now exists that permits growth and control of differentiation of normal human epidermal keratinocytes in defined medium. This culture system is a powerful

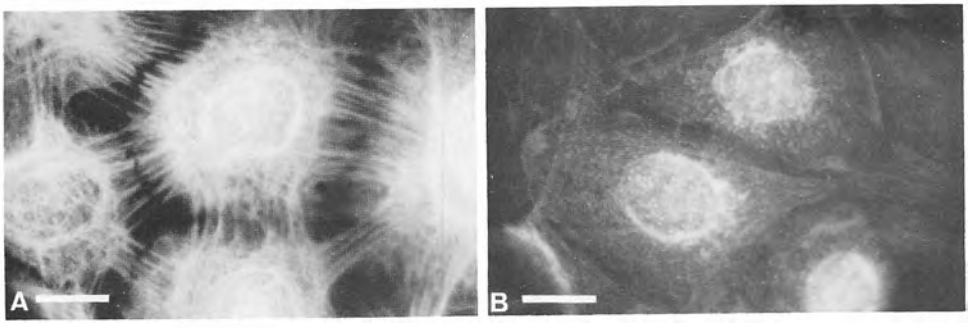


FIG 12. Indirect immunofluorescence of triton-extracted cells using rabbit antiserum against human keratin proteins and fluorescein labeled goat antiserum against rabbit IgG. The nuclei are labeled with propridium iodide. *A*, HK cells grown in defined medium. *B*, Swiss 3T3 cells grown in DME plus 10% dialyzed fetal bovine serum. Bars = 10 μm.

instrument for the study of a variety of medical and biological questions.

The authors wish to thank Dr. James G. Rheinwald for providing the rabbit antiserum against epidermal keratins, Mr. John Lye for assistance with indirect immunofluorescence, and Ms. Karen Brown for preparing the manuscript.

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