CULTIVATION, FROZEN STORAGE, AND CLONAL GROWTH OF NORMAL HUMAN EPIDERMAL KERATINOCYTES IN SERUM-FREE MEDIA

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SUMMARY: Methods are described for serum-free culture of human epidermal keratinocytes derived from neonatal foreskin tissue. Cultures are initiated, stored frozen, and returned to active growth, all with bovine pituitary extract as the only undefined supplement. Clonal growth assays are then performed in a biochemically defined medium. The degree of stratification and differentiation in the defined medium (and also with pituitary extract) is controlled by the extracellular calcium ion concentration.

Key words: keratinocytes; human; epidermis; serum-free; calcium; differentiation.

I. INTRODUCTION

Advances in the treatment of epidermal skin diseases require model systems to study the mechanisms of these disorders. Understanding the pathological state, in turn, relies heavily on understanding the normal biology of the skin. Although animal models are useful for study of both the normal and pathological biologies of the skin, it is often difficult to determine in animals whether abnormalities in individual cells of the epidermis are the cause or effect of an observed condition. Alternatively, pure strains of normal human epidermal cells in culture offer a highly desirable experimental system that allows precise manipulation of the cellular environment and more definitive interpretation of cellular responses. Methods are described here for culture in serum-free media of the predominant cells of healthy human epidermal tissue, the keratinocytes.

Cultured epidermal keratinocytes of murine origin have been used extensively to study carcinogenesis in vitro (34). However, because the cellular responses of murine keratinocytes may not always be equivalent to those of human cells, and because murine models for human epidermal diseases are not always available, the usefulness of murine keratinocytes in studies of human pathology is rather limited.

Cultured human keratinocytes (HK) have also been employed as model systems. Normal HK have been directly compared to clinically malignant epidermal carcinomas (25) and to psoriatic keratinocytes (17) by growth in culture. These in vitro techniques have already made significant contributions to the understanding of certain epidermal pathologies. However, in previous culture systems, broad application of cultured HK (10,17,25) to investigations of human diseases has been severely restricted by requirements for high cellular population densities, specialized substrates, or feeder interactions with fibroblastic cells. Growth-promoting substrates that have been used include collagen-coated dishes (10,16,17) and specially processed pigskin (6,24). Feeder interactions that have been employed include use of irradiated 3T3 cells (26) and an initial period of co-culture with human fibroblasts (20). Serial subculture in conventional basal media has been achieved by several means, including use of agents that increase intracellular cyclic AMP levels (9), use of fibronectin-coated culture dishes (7,8), and inoculation with small aggregates of keratinocytes rather than single cells (20).

Previous contributions from this laboratory have identified qualitative and quantitative nutritional requirements for normal human epidermal keratinocytes that have reduced (22,23) and then eliminated (30) the need for biochemically undefined supplements in the culture medium. However, the wide-scale use of these culture techniques has been restricted in part by the need to use a feeder layer for the primary culture and by inability to store the cells frozen. Therefore, three sets of improved media and techniques have been developed: the first for initiation of cultures without a feeder layer, and with whole bovine pituitary extract (wBPE) as the only undefined supplement; the second for frozen storage and recovery, again with wBPE; and the third for improved clonal growth and differentiation assays that employ only biochemically defined supplements.

The conditions that were developed for primary cul-

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ture of HK with pituitary extract select for highly viable unstratified cell populations. Cultures grown in these conditions provide an inoculum that can be subcultured at clonal cell density (25 cells/cm^2) with high (30%) colony-forming efficiency and a rapid growth rate (1 population doubling/day). These culture conditions permit at least seven serial subcultures and approximately 40 cumulative population doublings (2).

The second set of conditions allows HK to be routinely stored frozen in liquid nitrogen, recovered into culture with high viability, and subsequently used as effectively in experiments as cells that had never been frozen.

The third set of conditions employs a biochemically defined culture medium, consisting of basal nutrient medium MCDB 153 supplemented with epidermal growth factor (5 ng/ml), bovine insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), ethanolamine (0.1 m*M*), and phosphoethanolamine (0.1 m*M*), that supports rapid growth of HK cells. Adjustment of extracellular calcium ion concentrations between 0.03 and 1.0 m*M* in this defined medium has profound effects on growth, morphology, and stratification of HK colonies. These calcium-dependent effects correspond to a reciprocal relationship between growth rate (population doublings/day) and the degree of epidermal differentiation (percent cells with cornified envelopes) in vitro (2).

II. MATERIALS

A. Equipment

Cell culture incubator: water-jacketed, 37° C, 5% CO_2, saturated humidity
Microscope: inverted, phase contrast, with long working-distance condenser
Dissecting instruments: fine mouse-tooth and needle-point forceps, small scissors
Reverse osmosis unit, RO-4, Millipore[1]
Milli-Q water purification system, Millipore[1]
Blender: Waring, commercial grade, one quart, Dynamics Corporation of America[2]
Refrigerated centrifuge: Sorvall RC-5, Dupont[3]

B. Chemicals and media

All biochemicals and hormones (except as noted otherwise): "Sigma grade", Sigma Chemical Company[4]
Trace element salts (except sodium silicate): Specpure grade, Johnson Matthey Chemicals Limited[5]
All other inorganic chemicals: ACS reagent grade, Fisher Scientific[6]
HEPES buffer: Research Organics[7]
Epidermal growth factor (EGF): culture grade, Bethesda Research Labs[8]
Insulin: bovine, crystalline, Sigma
Trypsin: bovine, chromatographically purified, Type III, Sigma
Soybean trypsin inhibitor: type I-S, Sigma
Collagenase: type I, Worthington[9]
Collagen: Vitrogen 100, Collagen Corporation[10]
Disinfectant: Dettol, Reckitt and Coleman Pharmaceutical[11]
Antibiotic-antimycotic: penicillin, streptomycin, fungizone, GIBCO[12]
Solution A, 30 m*M* HEPES-NaOH buffer, 10 m*M* glucose, 3 m*M* KCl, 130 m*M* NaCl, 1 m*M* $Na_2HPO_4 \cdot 7H_2O$, 0.0033 m*M* phenol red, pH = 7.4 (*see* Procedure B).
Medium MCDB 153, consisting of MCDB 151 (23) with the following changes and additions in its trace element composition: copper 11 n*M*, iron 5 μ*M*, manganese 1 n*M*, molybdenum 7 n*M*, nickel 0.5 n*M*, selenium 30 n*M*, silicon 0.5 μ*M*, tin 0.5 n*M*, vanadium 5 n*M*, zinc 0.5μ*M* (*see* Procedure C). The complete formulation of medium MCDB 153 can be found in Table 1 of reference (3).
Whole bovine pituitary extract (wBPE), prepared fresh from freshly collected pituitaries and stored frozen at -20° C (*see* Procedure E).

C. Other supplies

Glass bottles, Wheaton[13]
Plastic bottles, Nalge[14]
Glass pipettes, Bellco[15]
Plastic (polystyrene) tubes, Falcon, Becton Dickinson[16]
Plastic tissue culture flasks, Corning[17]
Plastic tissue culture dishes, Lux, Miles[18]
Sterilization filters, Millipore[1]
Freezing ampules, Nunc, CryoMed[19]

III. PROCEDURES

A. Water

For all media and solutions that will come into contact with cultured cells, use high purity deionized water (or other high purity water that has been tested and found satisfactory for serum-free cell culture). In this laboratory, tap water that has been passed sequentially through reverse osmosis (Millipore RO-4) and the Milli-Q water purification system is used.

B. Solution A

1. Dissolve 0.125 g of phenol red (sodium salt) in 100 ml of water to prepare a 1000x stock solution. Store at room temperature in a tightly stoppered bottle.

2. Dissolve the following in approximately 900 ml of high purity water: HEPES, 7.149 g; glucose, 1.802 g; KCl, 0.224 g; NaCl, 7.697 g; $Na_2HPO_4 \cdot 7H_2O$, 0.268 g; phenol red 1000x stock, 1.0 ml.

3. Adjust pH to 7.4 with 4.0 *N* NaOH and volume to 1.00 liter with high purity water.

4. Sterilize with a detergent-free 0.2-μm pore size membrane filter and store at 4° C until used. If large amounts of solution A are used, a 10x concentrate can be prepared and diluted to final volume as needed.

C. Medium MCDB 153:

1. Prepare stocks 4a, 4b, and 4c as described in Table 1. These stocks will replace stock 4 of MCDB 151.
2. Prepare stock L (trace elements) from medium MCDB 104 as described in reference (19). This stock will replace stock 11 of MCDB 151.
3. Prepare medium MCDB 151 without stocks 4 (calcium chloride, magnesium chloride, ferrous sulfate) and 11 (zinc sulfate) as described in Tables 1–13 of reference (23), except reduce the final volume from 989 to 983 ml to accommodate the larger volume of stocks to be added. Store frozen at -20° C for up to 6 mo. (Do not prepare stocks 4 and 11 of MCDB 151. They will be replaced with stocks 4a, 4b, 4c, and L.)
4. Prepare complete medium MCDB 153 in small amounts just before it is to be used. (If necessary the complete medium can be stored at 4° C for up to 1 wk.)
   a. To each 98.3 ml of MCDB 151 minus stocks 4 and 11, the following are added with stirring:
      1. Stock 4a, 0.10 ml;
      2. Stock 4b, 0.10 ml;
      3. Stock 4c, 0.50 ml;
      4. Stock L, 1.0 ml.
   b. Sterilize the completed medium by filtration with a detergent-free 0.2-μm pore sized membrane filter. Filter the medium promptly after addition of stock 4c to avoid possible loss of precipitated iron on the sterilizing filter. Alternatively, add sterile stock 4c aseptically after the medium has been filter sterilized.

D. Defined supplements (1000x stocks)

1. Redissolve sterile lyophilized EGF in sterile Solution A either at 5 μg/ml or at 10 μg/ml. The former is a 1000x concentrate for clonal growth experiments and the latter a 1000x concentrate for stock cultures. EGF should not be refiltered after being redissolved due to its low protein content.
2. Dissolve insulin in aqueous 12 m*M* HCl at 5 mg/ml, which yields a solution whose pH is approximately 2, and sterilize by filtration. This concentration of insulin will precipitate at neutral pH, and therefore cannot be prepared in Solution A.
3. Dissolve hydrocortisone in absolute ethanol at 0.5 mg/ml, and sterilize by filtration.
4. Dissolve ethanolamine (2-aminoethanol) and phosphoethanolamine (o-phosphorylethanolamine) separately in Solution A at a concentration of 0.1 *M*, and sterilize each by filtration.
5. Store the sterile stocks of defined supplements in 0.5 ml aliquots in sterile snap-capped plastic tubes at -20° C.

E. Whole bovine pituitary extract (wBPE)

1. Prepare saline solution (0.15 *M* NaCl) by dissolving 8.768 g NaCl in 1.00 liter of high purity water.
2. Make arrangements to obtain fresh mixed sex pituitaries from cattle slaughtered at approximately 18 mo. of age. Be certain that the entire pituitary gland, which is partially embedded in a recess in the skull, is collected. Chill the pituitaries on ice immediately after removal and keep them cold at all times. For best results, prepare the pituitary extract within a few hours.
3. Rinse off excess blood with cold water and keep the pituitaries in a beaker of chilled saline on ice except when handling them. Place several thicknesses of paper towels on a cutting board. With a new scalpel blade, trim off the excess connective tissue, blood vessels, and adherent blood clots. Place the trimmed pituitaries in a fresh beaker of saline on ice.

TABLE 1

DIVALENT CATION STOCK SOLUTIONS FOR MCDB 153[a]

	Concentration in Stock Solution		Dilution into Final Medium	Concentration in Final Medium	
Stock component	g/l	*M*/l		mg/l	M/l
4a $CaCl_2 \cdot H_2O$	4.411	3.0E-2	1:1000	4.411	3.0E-5
4b $MgCl_2 \cdot 6H_2O$	122.0	6.0E-1	1:1000	122.0	6.0E-4
4c $FeSO_4 \cdot 7H_2O$	0.278	1.0E-3	1:200	1.39	5.0E-6

[a] Dissolve the components of stocks 4a and 4b in water and store tightly stoppered at room temperature. Stock 4c must be stored at acid pH. Dissolve solid in 2.5 ml 4 *M* HCl. Dilute to 1000 ml with H_2O. Filter sterilize and store at room temperature in a tightly closed bottle. Discard if orange precipitate forms. Molar concentrations are given in computer style exponential notation (1.0E-3 = 1.0×10^{-3}).

4. When approximately 40 to 50 pituitaries have been trimmed, start the first blender run. Rinse the trimmed pituitaries with saline and drain thoroughly. Weigh out no more than 105 g of pituitaries and place in a chilled blender head. Add chilled saline (250 ml/105 g of pituitaries), homogenize the pituitaries at maximum blender speed for 10 min. This should be done in a cold room or refrigerated chamber to minimize heating. If multiple runs are done, allow the blender motor to cool thoroughly between runs, or use different motors for alternate runs. Heating at this stage is very harmful.

5. Transfer the homogenate to a large beaker in a plastic pan of ice. Place a large magnetic stirring bar in the beaker and set the pan of ice with the beaker on a magnetic stirrer in a cold room or cold chamber. Stir the homogenate vigorously for at least 90 min. If multiple homogenizer runs are done, add each new homogenate as it is prepared. Stir vigorously for at least 90 min after the last batch is added. Occasionally use a spatula to mix the foamy upper part that does not stir well with the magnetic stirrer.

6. Divide the extract into screw-capped centrifuge tubes and balance them. Centrifuge at 13,200 × *g* for 10 min at 4° C.

7. Decant the supernatants and combine them in a beaker chilled on ice. Pass the supernatant through Whatman #1 filter paper in a porcelain Buchner funnel on a suction flask. Change filters each time the flow rate declines. Combine the filtrates, dispense them into plastic bottles of 100 ml or less, and store frozen at -20° C until needed.

8. Thaw a bottle of frozen extract. Centrifuge and filter with Whatman #1 paper again as described in steps 6 and 7 above. Pass the extract successively through detergent-free membrane filters with 0.8, 0.45, and 0.2 μm pore sizes. Use sterile filters and receiving vessels for the final filtration. Dispense the sterilized extract into 1 or 2-ml tubes and store frozen at -20° C until used. These small aliquots can be thawed and refrozen several times with little loss of potency. The frozen extract can be stored at -20° C for at least 6 mo.

9. The protein concentration of the wBPE measured by the methods of Lowry et al. (18) with bovine serum albumin as a standard will normally be about 14 mg/ml. (Concentrations of wBPE used in growth media are specified in terms of protein for purposes of standardization, although the possibility remains that nonprotein components may be contributing to growth-promoting activity.)

F. Preparation of complete media for primary culture, freezing, and clonal growth

Add the appropriate supplements to MCDB 153 in the amounts indicated in Table 2. Note that ethanolamine and phosphoethanolamine are used at concentrations that are 10 times greater than those previously employed for human keratinocytes (30).

G. Antibiotics

Add 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml Fungizone to all HK cultures (optional after establishment of primary cultures).

H. Isolation and primary culture of human keratinocytes

1. Make arrangements with a hospital newborn nursery to obtain human foreskin tissue removed during routine circumcision of newborn male infants. Immediately after excision, place the foreskins in Solution A plus penicillin, streptomycin, and Fungizone and store at 4° C. Initiate cultures no longer than 48 h after circumcision. Figure 1 is a flow diagram of the primary culture procedure. Perform all steps described below under sterile conditions.

2. Place each foreskin in a petri dish of sterile Solution A and trim the larger pieces of subcutaneous connective tissue from the dermal surface.

3. Wash the tissue 15 to 30 s in 5% (vol/vol) Dettol. Rinse by dipping the tissue in three separate dishes of sterile Solution A.

4. Keeping the tissue submerged in Solution A, cut it into pieces approximately 4-mm square.

5. Transfer the tissue pieces into 15 ml of a solution of 625 U/ml collagenase plus 10% (vol/vol) wBPE in MCDB 153 containing 0.1 m*M* Ca^{2+}. (Note that collagenase in calcium-free saline will not adequately digest the tissue. wBPE is added to the digestion solution to provide protein substrates for proteases not specific for collagen that are present in this collagenase preparation. If strictly serum-free conditions are not essential, 10% fetal bovine serum can be used in place of 10% wBPE.) Transfer 7.5 ml of the suspension to each of two 60-mm petri dishes and place into a 37° C incubator with 5% CO_2 and saturated humidity for 90 to 120 min or until epidermis is readily removable from dermis.

6. While collagenase digestion is in progress, prepare tissue culture flasks of 25, 75, or 150-cm^2 area. A typical preparation requires 150 cm^2 of culture surface and 25 ml of supplemented medium per foreskin dissected. For each 100 ml of supplemented stock culture medium (Table 2), sterilely add together 98 ml MCDB 153, 0.07 ml $CaCl_2$ (0.1 *M*), 0.1 ml EGF (10 μg/ml), 0.1 ml insulin (5 mg/ml), 0.1 ml hydrocortisone (0.5

TABLE 2

SERUM-FREE SUPPLEMENTS FOR STOCK CULTURE, FROZEN STORAGE, AND CLONAL GROWTH OF HUMAN KERATINOCYTES

	Stock Cultures	Frozen Storage	Clonal Growth
EGF	10 ng/ml	10 ng/ml	5 ng/ml
Insulin	5 μg/ml	0	5 μg/ml
Hydrocortisone	1.4 μ*M* (0.5 μg/ml)	1.4 μ*M* (0.5 μg/ml)	1.4 μ*M* (0.5 μg/ml)
Ethanolamine	0.1 m*M*	0	0.1 m*M*
Phosphoethanolamine	0.1 m*M*	0	0.1 m*M*
$CaCl_2$ (final concentration)	0.1 m*M*	0.1 m*M*	0.03 to 1.0 m*M*
wBPE	70 μg/ml (0.5% vol/vol)	700 μg/ml (5% vol/vol)	0
DMSO	0	10% (vol/vol)	0

mg/ml), 0.1 ml ethanolamine (0.1 *M*), 0.1 ml phosphoethanolamine (0.1 *M*), 0.5 ml wBPE (14 mg/ml protein), and 1.0 ml of 100X penicillin-streptomycin-Fungizone stock solution. Gently mix the medium after each addition. The medium should not be refiltered after the supplements have been added because the small amounts of EGF and insulin are likely to be adsorbed onto the filter. Add 25 ml of supplemented medium to each 150-cm^2 flask, 15 ml to each 75-cm^2 flask, or 5 ml to each 25-cm^2 flask. Place the flasks into a 37° C incubator gassed with 5% CO_2 in air saturated with water vapor. Loosen the caps on the flasks to allow the medium to equilibrate with the 5% CO_2.

7. Remove the collagenase digestion mixture from the 37° C incubator after 90 to 120 min. Carefully aspirate the collagenase solution away from the tissue pieces and replace it with Solution A. For each piece of tissue, remove the epidermis from the dermis by holding the dermis with fine mouse-tooth forceps and pulling the epidermis from the edge with needle-tipped forceps. The epidermis should separate as an intact sheet. Sterilize the forceps by dipping them in 95% ethanol and burning off the ethanol.

8. As each piece of tissue is separated, place the epidermal layer in a 60-mm petri dish containing Solution A at room temperature. The dermis is

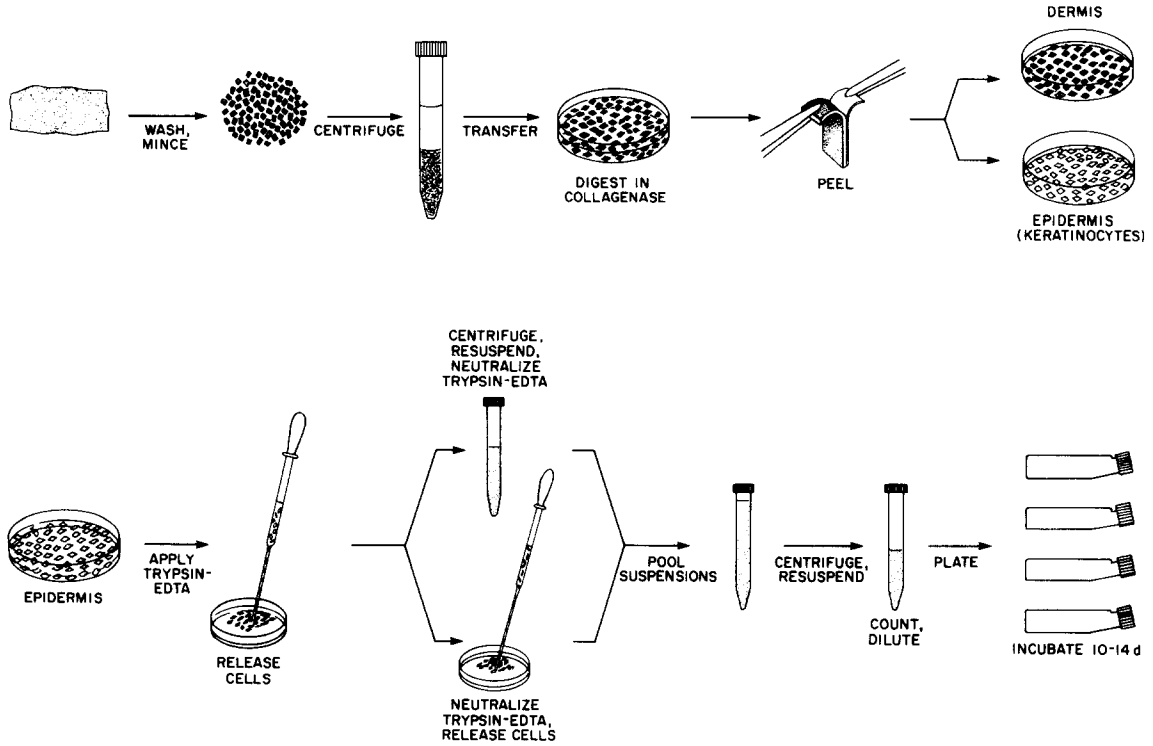

FIG. 1. Flow diagram of procedures for establishment of cultures of normal human epidermal keratinocytes from neonatal foreskin. Reprinted with permission from In Vitro Models for Cancer Research, Webber, M.; Sekely, L., eds., vol. 3. CRC Press Inc.; 1985, in press Boca Raton, FL.

discarded, unless it is used to culture dermal cell types. Collect the epidermal sheets from all of the tissue fragments.

9. Gently aspirate off the Solution A, leaving the epidermal sheets in the dish. Add 6 ml of a solution of 0.025% trypsin (wt/vol) and 0.01% EDTA (wt/vol) in Solution A (pH 7.4). Gently agitate the epidermal fragments up and down in a cotton-plugged, sterile Pasteur pipette for 3 to 4 min to release individual cells. Note that prolonged exposure of the cells to the trypsin-EDTA mixture reduces the yield of viable cells. Using a sterile Pasteur pipette, withdraw the cell suspension from the remaining tissue fragments, place the suspension in a sterile centrifuge tube, and centrifuge at 250 × *g* for 5 min.
10. Add 6 ml of a solution containing 0.1% (wt/vol) soybean trypsin inhibitor (STI) plus 10% (vol/vol) Vitrogen 100 in MCDB 153 to the epidermal pieces left in the petri dish. Alternatively, if strictly serum-free conditions are not essential, 10% whole fetal bovine serum may be used in place of 0.1% STI plus 10% Vitrogen 100. Also resuspend the pellet from the centrifugation in 6 ml of the same solution by gentle pipetting until it is broken into many small clumps. Release more individual cells from the epidermal pieces in the petri dish by repeating the agitation process described in step 9 for an additional 5 min. Transfer the resulting cell suspension (but not the remaining tissue fragments) from the dish to the tube containing the resuspended pellet and centrifuge at 250 × *g* for 5 min.
11. Aspirate the trypsin-inhibitor or serum solution from the pellet, resuspend the pellet in 2 to 3 ml of the supplemented stock culture medium, and gently pipette until the pellet is dispersed into a suspension of single cells (with some small clumps remaining). Count the cells with a standard hemocytometer (or other suitable device) and inoculate the cells into the pre-equilibrated flasks (step 6) at a density of 3 to 4 × 10^3 cells/cm^2.
12. Shake the inoculated flasks gently to distribute the cells evenly and incubate them at 37° C in an atmosphere of 5% CO_2 in air saturated with water vapor, with the caps loosened for equilibration. After 48 h, gently remove the medium and replace it with fresh medium of the same composition, without agitating or rinsing the cultures. At 5 to 7 d after inoculation, remove the medium, wash the cultures twice with Solution A, and add fresh medium. Repeat this procedure again at 10 to 12 d after inoculation (or 24 h before harvest of the primary cultures).
13. Cultures are considered "mature" and ready for subculture or freezing when they are approximately half confluent and satellite colonies of two to eight cells begin to appear (*see* Discussion for details).

I. Frozen storage and subculture

1. Prepare the freezing medium in advance. For serum-free frozen storage, use medium MCDB 153 supplemented with 5% (vol/vol) wBPE, 0.1 m*M* Ca^{2+} (final concentration), 10 ng/ml EGF, 0.5 μg/ml hydrocortisone, and 10% (vol/vol) DMSO (Table 2). Alternatively, if serum-free conditions are not essential, MCDB 153 supplemented with 20% FBS, 0.5% (vol/vol) wBPE, 0.1 m*M* Ca^{2+}, 10 ng/ml EGF, 0.5 μg/ml hydrocortisone and 10% DMSO can be used with good results.
2. Aspirate the growth medium from mature primary cultures, and wash the cells twice with Solution A.
3. Cover the cells with 0.025% trypsin (wt/vol) and 0.01% EDTA (wt/vol) in Solution A (pH 7.4). Use 2 ml/25-cm^2 or 75-cm^2, or 4 ml/150-cm^2 flask. After 30 to 60 s, remove as much as possible of the solution by aspiration and leave the flasks at room temperature with their caps screwed down tightly to prevent evaporation of the thin film of trypsin-EDTA solution still covering the cells.
4. Examine the cultures with an inverted microscope after 3 to 4 min. If less than 50% of the cells have detached, allow the digestion to continue for successive 3-min periods until more than 50% of the cells are detached. When this point is reached, a gentle rap against a hard surface will release almost all of the cells from the culture surface.
5. Resuspend the detached cells in 5 ml of freezing medium. Centrifuge at 250 × *g* for 5 min. (If cells from several flasks are being frozen, pool them and centrifuge to form a single pellet.)
6. Resuspend the pelleted cells in 2 to 3 ml of freezing medium, disaggregate clumps by gentle pipetting, and count the cells. The cell density is adjusted to 2 to 4 × 10^5 cells/ml and 1-ml aliquots are distributed into 1.2-ml capacity plastic screw-capped freezing ampules.
7. Freeze the ampules at a controlled rate (4) from room temperature to -85° C. In this laboratory, controlled rate freezing is accomplished by placing the ampules upright into holes in a styrofoam block that has been cut in half, such that each ampule is surrounded on all sides by at least 7 mm of styrofoam, sealing the seam in the block with tape, and placing the block into a -85° C freezer for 12 to 24 h. After the ampules have reached -85° C, remove them from the

block and quickly transfer them to liquid nitrogen for long-term storage.

8. Initiate secondary cultures from the frozen suspensions and grow them in the same medium and under the same conditions as described for primary cultures.

   a. Remove frozen ampules from liquid nitrogen and thaw at 37° C in 70% EtOH. Eye protection should be worn when handling ampules of frozen cells because they may explode if liquid nitrogen has leaked into them.

   b. Inoculate cells at a density of 500 to 3000 cells/cm^2 into flasks containing preincubated stock culture medium. Change the medium after 24 h, and either subculture again or prepare inocula for clonal growth experiments after 4 to 7 d. Use the same criteria of culture maturity (*see* Discussion) as for primary cultures. To encourage confluent cultures, change the medium daily.

9. For subculturing without freezing, resuspend the cells from step 3 in stock culture medium (Table 2), centrifuge, resuspend again in stock culture medium, and inoculate flasks at a density of 500 to 3000 cells/cm^2, as described in step 7b.

J. Clonal growth in defined medium

1. The standard defined medium for clonal growth of HK consists of MCDB 153 supplemented with 5 ng/ml EGF, 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, 0.1 m*M* ethanolamine, and 0.1 m*M* phosphoethanolamine (Table 2). In many cases, the Ca^{2+} concentration in the medium is increased to 0.1, 0.3, or 1.0 m*M*, (*see* Discussion) by adding appropriate volumes of 0.1 *M* $CaCl_2$ in water, taking into account the basal level of 0.03 m*M* Ca^{2+} that is already present in MCDB 153.

2. Prepare the test media that are to be used in the clonal growth assays (either the standard medium described above or experimental variations). Dispense the media into petri dishes (4.9 ml/60-mm dish), and preincubate the dishes containing media in the cell culture incubator for at least 1 h while preparing the cellular inoculum.

3. Release cells from their culture surface with trypsin-EDTA as described in the "Frozen Storage" section. Resuspend the detached cells either in 10% FBS in MCDB 153 or in 0.1% soybean trypsin inhibitor plus 10% Vitrogen 100 in Solution A and then centrifuge at 250 × *g* for 5 min. Remove the supernatant by aspiration and resuspend the pellet in 2 to 3 ml of unsupplemented MCDB 153. Disaggregate the pellet to single cells by gentle pipetting.

4. Count and dilute the cells in unsupplemented MCDB 153 to 5 × 10^3 cells/ml, and add 0.1 ml (500 cells) of the cell suspension to each preincubated 60-mm dish containing 4.9 ml of test medium.

5. Incubate the cultures at 37° C in an atmosphere of 5% CO_2 in air saturated with water vapor for 10 to 14 d without medium change. Remove the medium, fix the colonies 10 to 15 min with 2% (vol/vol) glutaraldehyde buffered with 0.05 *M* sodium cacodylate (pH 7.0), and stain the cells with 0.1% crystal violet.

IV. DISCUSSION

Improved culture conditions

The starting point for the development of improved culture conditions for normal human epidermal keratinocytes was a system described previously (30) for clonal growth of HK in a defined medium consisting of MCDB 152 supplemented with 5 ng/ml EGF, 5 μg/ml insulin, 1.4 μ*M* (0.5 μg/ml) hydrocortisone, 10 μg/ml transferrin, 2 n*M* progesterone, 0.01 m*M* ethanolamine, and 0.01 m*M* phosphoethanolamine. Although that system yielded good cellular growth in the absence of serum or other undefined supplements, it was necessary to begin with an inoculum prepared from a primary culture grown by a modification of the Rheinwald and Green (26) feeder layer technique in MCDB 152 with 20% FBS and 10 μg/ml hydrocortisone.

The experimental procedures described in Materials and Procedures of this report now make possible isolation and multiplication of primary cultures, frozen storage, multiplication of secondary cultures after thawing, and clonal growth assays, all without use of a feeder layer and, if required, without exposure to serum at any point during the procedures.

Primary cultures. The procedure described for separating epidermis from dermis, which is modified from a procedure described by Yuspa and Harris (33) in which trypsin rather than collagenase was used, results in a primary inoculum of keratinocytes that contains relatively few fibroblastic cells. The cell yield from the primary isolation ranges from 2 × 10^5 to 1 × 10^6 cells per foreskin, and the procedure is simple enough to permit several foreskins to be pooled in a single large culture. Typically, a primary isolation of keratinocytes from 4 to 6 foreskins yields a total of 1 to 3 × 10^6 cells. Initial colony-forming efficiency is approximately 0.1 to 3.0%.

The primary culture conditions that have been developed suppress fibroblastic cells while enhancing proliferation of keratinocytes, thus allowing elimination of the 3T3 feeder layer. Cultures of HK inoculated at the cell densities described and grown in the stock culture medium (Table 2) with 0.1 m*M* Ca^{2+} form nonstratified monolayer colonies with a rounded, packed appearance (Fig. 2). The cells tend to stop

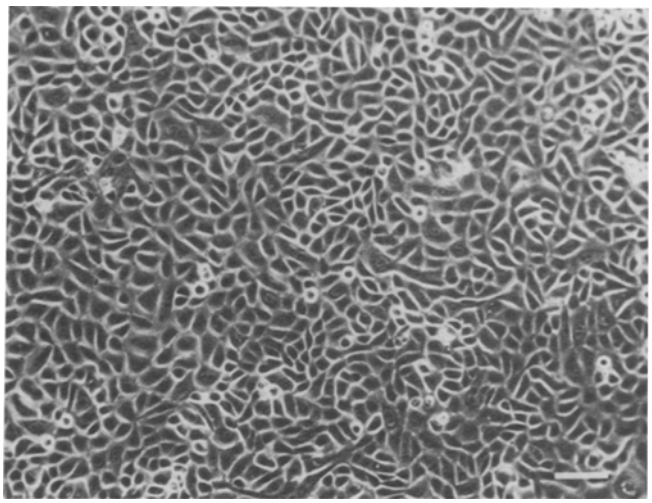

FIG. 2. Phase contrast micrograph of HK culture showing confluent sheet of HK cells within a large colony before frozen storage. Note that many mitotic cells are present. Scale bar = 0.1 mm.

multiplying before reaching full confluency unless the medium is changed daily. Primary cultures are judged to be "mature" and ready for harvesting when they are about half confluent and two to eight cell satellite colonies begin to appear, but many mitotic cells remain visible in the larger colonies. This degree of development ordinarily requires 10 to 14 d.

For best subsequent growth, it is important to freeze or subculture the primary cultures while at least some mitotic cells are still visible in the initial colonies. Mature primary cultures initiated from five average-sized foreskins will contain a total of approximately 2 to 4 $\times$ 10^6 cells that can be subcultured with a high (30 to 40%) colony-forming efficiency (2).

It should be noted that the stock culture medium and the freezing medium are serum-free, but that they contain wBPE and are therefore not biochemically defined. The wBPE is distinctly beneficial to the cells during the establishment of primary cultures and also for frozen storage and recovery of highly viable cells. There is also a slight benefit from the addition of wBPE during clonal growth assays with subcultured cells (2), but the effect is much smaller and good clonal growth can be obtained readily without undefined supplements.

Clonal growth assay. In previous studies (30), at least 2000 cells/60-mm petri dish (100 cells/cm^2) were needed for satisfactory clonal growth with defined supplements. With the procedures described here, an inoculum of 500 cells per dish (25 cells/cm^2) can be used routinely, although the clonal growth experiment illustrated in Fig. 3 was performed with 1000 cells per dish (50 cells/cm^2). The improved colony-forming efficiency is due primarily to the use of a cellular inoculum prepared from cultures that are free of stratification and terminally differentiated cells (equivalent to Fig. 2).

Regulation of differentiation. Control of keratinocyte differentiation in culture by adjustment of calcium ion concentration has been reported both for human keratinocytes (10,23,30,31) and for mouse keratinocytes (11). The ability of this single variable to regulate expression of keratinocyte differentiation under fully defined conditions is demonstrated in Fig. 3. Stepwise increase in calcium concentration from 0.03 to 1.0 m*M* (from left to right in Fig. 3) causes distinct changes in colony morphology and total colony growth that seem to be directly related to the extent of stratification and terminal differentiation. Also, at 1.0 m*M* Ca^{2+}, the defined medium becomes less selective against fibroblasts, such that diffuse colonies of apparently stressed fibroblasts are sometimes seen (Fig. 3 *D*, *upper row*).

These changes can be seen at higher magnification in the *lower row* of Fig. 3. At 0.03 m*M* Ca^{2+}, the basal concentration in MCDB 153, the colonies are very flat with a spreading morphology and no tendency for the polygonal cells to stratify or exhibit any major degree of terminal differentiation (Fig. 3 *A*, *lower row*). (Note that some enlarged cells are seen.)

At 0.1 m*M* Ca^{2+} the colonies are still predominantly monolayers, but the cells pack together much more tightly and small foci of stratification, 2 to 3 cells deep, begin to appear. The peripheries of the colonies are continuous, but exhibit an irregularly winding shape (Fig. 3 *B*, *lower row*).

At 0.3 m*M* Ca^{2+} the cells become very tightly packed together and the colony borders become very irregular with deep invaginations of varying size (Fig. 3 *C*, *lower row*). Substantial areas of stratification are present, with cornified envelopes on the apical cells that resist penetration of stain and that fail to dissolve when heated to boiling in the presence of sodium dodecyl sulfate and dithiothreitol (29).

At 1.0 m*M* Ca^{2+} clonal growth in the defined medium produces very small, densely packed colonies that are highly stratified and exhibit cornified envelopes in their apical cells (Fig. 3 *D*, *lower row*).

Historically, normal human epithelial cells have been very difficult to culture and it is only in recent years that a limited number of methods for their culture have become available. Therefore, the development and characterization of a cell culture system for a normal human epithelial cell type that uses a defined medium and that is easily reproducible provides a valuable model for study of human epithelial biology at the cellular and subcellular levels. More specifically, this model system is already making a significant contribution to the detailed study of human epidermal pathologies.

Biomedical Applications

According to the Health and Nutritional Examination Survey (14) in which 20,749 individuals were examined, nearly one-third had one or more conditions that the dermatologist considered a "significant" di-

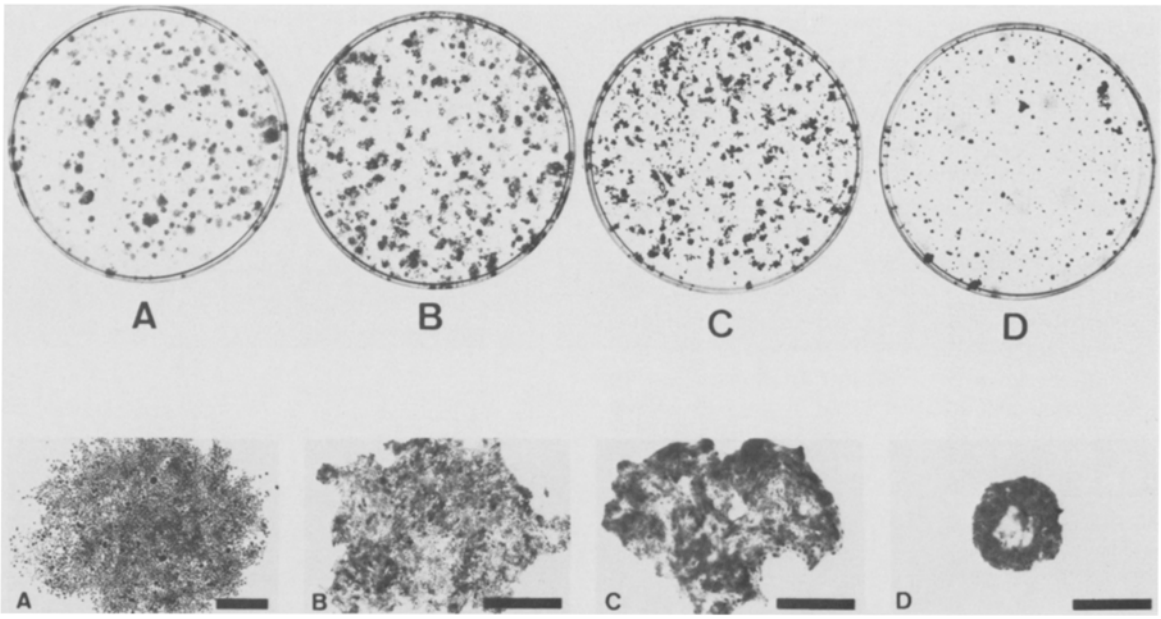

FIG. 3. Effect of calcium concentration on growth rate and colony morphology. Clonal growth assays were performed in defined medium as described in Methods and Materials, with the following calcium ion concentrations in the vertical columns, left to right: *A*, 0.03 m*M*; *B*, 0.1 m*M*; *C*, 0.3 m*M*; and *D*, 1.0 m*M*. The cellular inoculum was 1000 cells/60-mm dish (50 cells/cm^2), derived from a serum-free secondary culture after recovery from frozen storage. The colonies were stained with crystal violet. *Top:* Photographs of entire dishes. Note the presence of a few poorly growing diffuse colonies of fibroblastlike cells at the highest calcium concentration (*top,D*) and their absence at lower calcium concentrations. *Bottom*, photographs of individual colonies. Scale Bar = 1.0 mm. Note the failure of the stain to penetrate cornified envelopes, particularly at the highest calcium concentration (*bottom,D*).

agnosis. The serum-free culture system for HK cells described here has already been used as a model system to investigate certain clinical disorders of the epidermis.

Epidermal cytotoxicity assays. Cytotoxic destruction of keratinocytes by effector cells of the immune system (monocytes, lymphocytes, neutrophils) contributes to the development of the clinical symptoms of several skin diseases. This cytotoxicity can occur by the direct action of the immune effectors on the keratinocyte targets (cell-mediated cytotoxicty, CMC), or it may occur only in the presence of an antibody that binds to the keratinocyte target, thereby identifying it for attack by the immune effectors (antibody-dependent cellular cytotoxicity, ADCC).

The culture system described here has been used to develop a model for in vitro study of the mechanisms of epidermal cytotoxicity (27) in which cultured HK were used as targets for nonsensitized immune effector cells. Antibody alone or antibody plus complement produced no significant lysis of HK targets. However, monocytes or lymphocytes, but not neutrophils, were capable of CMC of HK targets. Addition of a specific antibody against an antigen with which HK cells were coated also caused ADCC of HK cells by monocyte and lymphocyte effectors that was significantly higher than CMC by the same effectors.

In vitro induction of HLA-DR. Expression of human leukocyte antigens (HLA) of the DR locus is associated with the epidermis or its appendages in several human skin diseases including psoriasis, dermatitis herpetiformis, Sjogren's syndrome, graft versus host disease, pemphigus vulgaris, and lupus erythematosus (5). HLR-DR is also expressed strongly by dendritic cells of the epidermis called Langerhans cells, which are competent in a murine model, both to present antigen and to stimulate proliferation of allogeneic and syngeneic lymphocytes (1,28). Therefore, studies have been performed to determine if cultured HK that are known to be depleted of Langerhans cells can express HLA-DR antigens. Although initial reports (12,30) indicated that cultured HK do not express HLA-DR antigens, conditions have been identified in which HK cultured in defined medium containing gamma-interferon demonstrate expression of these antigens (13). These studies were extended to show that the expression of HLA-DR antigens results from their de novo synthesis by HK cultured in defined medium containing gamma-interferon (32). Further investigations of these kinds are anticipated to elucidate the role of HLA-DR expression by HK cells in the etiologies of certain skin diseases.

Ultraviolet light-induced binding of autoimmune antibodies. Another application for this culture method has shown that HK cells bind particular autoantibodies from serum of patients with the disease, lupus erythematosus (LE), after exposure in vitro to ultraviolet light (UVL) (15). Normal human serum showed no antibody

binding to the cultured cells after UVL exposure. An important clinical manifestation of LE is the occurrence of cutaneous lesions after exposure to the sun. These findings have helped to identify a subset of the autoantibodies associated with LE as candidates for participation in the formation of photosensitive cutaneous lesions of LE.

Future applications. The ability to perform detailed and extended studies on these and other epidermal disorders through use of a well characterized and convenient in vitro model system can be expected to provide a new understanding of the pathological mechanisms that are involved. This in turn should lead to major improvements in the treatment of these disorders and reduction of the suffering caused by them.

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