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CLONAL GROWTH OF HUMAN KERATINOCYTES WITH SMALL AMOUNTS OF DIALYZED SERUM[1]

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SUMMARY

A survey of commercially available media revealed that medium F-12 was superior to medium 199 for clonal growth of human epidermal keratinocytes (HK) when supplemented with 10 μg/ml hydrocortisone (HC) plus dialyzed fetal bovine serum protein (FBSP), rather than the whole serum used in previous studies. Qualitative and quantitative adjustment of the medium composition for optimal clonal growth with minimal amounts of FBSP generated a new medium, MCDB 151, which supports clonal growth of HK with 10 μg/ml HC and as little as 1 mg/ml FBSP (equivalent in protein concentration to 2.0% whole serum). MCDB 151 differs significantly from MCDB 105, previously developed in this laboratory for normal human fibroblasts, and each medium selectively favors growth of its own type of cell in primary cultures of disaggregated human neonatal foreskin cells. Differences in the amounts of calcium and adenine in the two media appear to be among the most influential factors mediating the selective growth. Optimal growth of HK occurs at a very low level of Ca^{2+} that causes the colonies to remain as monolayers rather than stratifying as they do in the presence of higher levels of calcium. However, keratin synthesis, which was examined through use of highly specific fluorescent antibodies, is not affected by the Ca^{2+} concentration. Agents that increase intracellular cyclic AMP levels appear to have no effect on HK growth in MCDB 151 with 10 μg/ml HC and 1.0 mg/ml FBSP.

Key words: keratinocytes; clonal growth; MCDB 151; dialyzed serum; calcium.

INTRODUCTION

In the accompanying paper (1), we showed that a fibroblast feeder layer or medium conditioned by fibroblasts was not required for the growth and differentiation of human epidermal keratinocytes (HK) if the proper culture conditions were provided. Those conditions included the use of medium 199 (2) supplemented with 10 μg/ml hydrocortisone (HC), 20% (v/v) whole fetal bovine serum (wFBS), and 0.15 mg/ml each of pituitary extract (PE) fractions, 3A and 3B. With such a supplemented medium, clonal growth, serial cultivation, and differentiation of HK were all possible in the complete absence of interaction with any other type of cell.

In order to determine more precisely the requirements for growth and differentiation of HK, it was essential to be able to culture the cells in a more defined system. Results with other cell types have revealed that the amounts of undefined macromolecular supplements needed for satisfactory multiplication can be reduced substantially by improvements in the synthetic portion of the medium (3). However, in order to make such improvements it is first necessary to be able to grow the cells with dialyzed serum so that the overall

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small molecular weight composition of the supplemented culture medium can be controlled accurately. Accordingly, growth of HK in various background media supplemented with dialyzed fetal bovine serum protein (FBSP) rather than whole serum was examined. Under these conditions, growth in medium 199 was poor, as it was also in most of the other media tested. However, medium F-12 (4) supplemented with HC, FBSP, and PE fractions supported a reasonable amount of clonal growth of HK.

This paper describes subsequent qualitative and quantitative adjustments that were made in the composition of medium F-12 and the reduction in the amount of FBSP needed for HK growth that resulted from such adjustments. In addition, comparison of the medium optimized for HK (MCDB 151) with a medium that previously had been optimized for fibroblasts (MCDB 105) (5) revealed that each was selective for growth of its own cell type and led to a study of the basis for that selectivity, which is also described in this paper.

Materials and Methods

Cells. Primary cultures of human keratinocytes were prepared as previously described (1). Unless otherwise indicated, primary suspensions of foreskin cells were inoculated with lethally irradiated 3T3 cells to prevent the attachment of dermal fibroblasts (6). On Day 3, the 3T3 cells were removed with EDTA as previously described (1). Stock cultures of the HK cells were routinely maintained in medium 199 supplemented with 10 μg/ml HC and 20% (v/v) wFBS and conditioned overnight by 3T3 cells. Cultures were incubated in a humidified atmosphere of 5% carbon dioxide and 95% air at 37° C in a laboratory whose altitude is 1650 m (5400 feet).

Subculture and plating experiments. Confluent cultures were resuspended as previously described (1) and used for subculture or plating experiments. Primary or secondary cultures were used in all clonal growth assays. The dishes were incubated undisturbed for 14 days, at which time they were fixed with 10% formalin and stained with Rhodanile Blue (6). Growth was compared visually or by counting the number of colonies with 16 or more cells.

Media and supplements. The following media were used: medium 199 with Hank's salts (powdered, GIBCO, Grand Island, NY); Dulbecco's modified Eagle's medium (DME, powdered, Flow Laboratories, Rockville, MD); RPMI 1640 (powdered, GIBCO); F-12 (powdered, GIBCO); modified L-15 (powdered, Flow Laboratories); modified McCoy's 5-A (powdered, GIBCO); Waymouth medium MB 752/1 (powdered, GIBCO); F-10 (powdered, GIBCO); modified minimal essential medium (MEM) with Hank's salts (powdered, Flow Laboratories); DME with 4.5 g/l glucose (powdered, GIBCO); MEM with Earle's salts (powdered, GIBCO); MCDB 104 (7); MCDB 105 (5); MCDB 401 (3); MCDB 411 (3); and MCDB 201 (8). The commercial powders were all prepared with 6.6 g/l HEPES

TABLE 1

Stock 1 (100X)

Component	Concentration in Stock Solution		Concentration in Final Medium	
	g/l	*mol/l*	*mg/l*	*mol/l*
Arginine·HCl	21.07	1.0E-1	210.7	1.0E-3
Histidine·HCl·H_2O	1.677	8.0E-3	16.77	8.0E-5
Isoleucine allo-free	0.1968	1.5E-3	1.968	1.5E-5
Leucine	6.56	5.0E-2	65.6	5.0E-4
Lysine·HCl	1.827	1.0E-2	18.27	1.0E-4
Methionine	0.4476	3.0E-3	4.476	3.0E-5
Phenylalanine	0.4956	3.0E-3	4.956	3.0E-5
Threonine	1.191	1.0E-2	11.91	1.0E-4
Tryptophan	0.306	1.5E-3	3.06	1.5E-5
Tyrosine	0.2718	1.5E-3	2.718	1.5E-5
Valine	3.513	3.0E-2	35.13	3.0E-4
Choline	1.396	1.0E-2	13.96	1.0E-4
Serine	6.306	6.0E-2	63.06	6.0E-4

Gentle heating and stirring is helpful in dissolving the components. The freshly thawed stock frequently contains a precipitate, which will dissolve on gentle warming. It is stored at 4° C for up to 2 months or frozen at −20° C for longer periods.

(2.8×10^{-2} *M*), and 1.2 g/l sodium bicarbonate (1.4×10^{-2} *M*). All were adjusted to pH 7.4 before addition of serum or PE. The MCDB media were prepared in this laboratory according to references (2,5,7,8), except that MCDB 105 was used in an atmosphere of 5% carbon dioxide instead of 2%.

For determining optimum concentrations of nutrients, medium F-12 was prepared as described in Appendix A of reference (9) with individual nutrients or groups of nutrients omitted. Those nutrients were added back from concentrated stock solutions in the amounts indicated. Media MCDB 150 and MCDB 151 were prepared by the same procedures as medium F-12 except for altered nutrient concentrations. Since a substantial number of nutrient concentrations were altered during its development, the complete procedure for preparation of MCDB 151 is described here.

MCDB 151 is prepared from a set of 13 stock solutions plus five components that are added directly to the medium as solids. Preparation of each of the 13 stocks is described in Tables 1-11 followed by procedures for preparing the final medium from the stocks (Tables 12, 13). Note that an abbreviated exponential notation is used for molar concentrations in which the letter E is used to designate "10 to the power." Thus, 1.0E-1 means 1.0×10^{-1} mol/l.

TABLE 2

STOCK 2 (100X)

Component	Concentration in Stock Solution		Concentration in Final Medium	
	g/l	*mol/l*	*mg/l*	*mol/l*
Biotin	0.00146	6.0E-6	0.0146	6.0E-8
Ca pantothenate	0.0258	1.0E-4	0.258	1.0E-6
Niacinamide	0.003663	3.0E-5	0.03663	3.0E-7
Pyridoxine · HCl	0.006171	3.0E-5	0.06171	3.0E-7
Thiamine · HCl	0.03373	1.0E-4	0.3373	1.0E-6
KCl	11.183	1.5E-1	111.83	1.5E-3

This stock is stored at 4° C up to 2 months or frozen at −20° C for longer periods.

TABLE 3

STOCK 3 (50X)

Component	Concentration in Stock Solution		Concentration in Final Medium	
	g/l	*mol/l*	*mg/l*	*mol/l*
Folic acid	0.0395	9.0E-5	0.79	1.8E-6
$Na_2HPO_4 \cdot 7H_2O$	26.81	1.0E-1	536.2	2.0E-3

This stock is stored at 4° C up to 2 months or frozen at −20° C for longer periods. The disodium phosphate provides alkaline conditions necessary to dissolve the folic acid. In experimental media where the folic acid is prepared separately, great care must be taken to be certain that the folic acid has dissolved. Under neutral or slightly acidic conditions folic acid tends to form a fine particulate suspension that is not easily seen in the medium but that is retained on the sterilizing filter. Once the folic acid has been dissolved completely, however, it normally will remain in solution in the final medium even under slightly acidic conditions.

TABLE 4

STOCK 4 (100X)

Component	Concentration in Stock Solution		Concentration in Final Medium	
	g/l	*mol/l*	*mg/l*	*mol/l*
$FeSO_4 \cdot 7H_2O$	0.0417	1.5E-4	0.417	1.5E-6
$MgCl_2 \cdot 6H_2O$	12.20	6.0E-2	122.0	6.0E-4
$CaCl_2 \cdot 2H_2O$	0.4411	3.0E-3	4.411	3.0E-5

A small amount of concentrated hydrochloric acid is added to the stock (1 drop/100 ml or 0.5 ml/l) to prevent the gradual precipitation of ferric hydroxides from the solution. This stock is stored in tightly stoppered bottles at room temperature. *Stock 4 is normally not added to the final medium until immediately before use.*

TABLE 5

STOCK 5 (1000X)

Component	Concentration in Stock Solution		Concentration in Final Medium	
	g/l	*mol/l*	*mg/l*	*mol/l*
Phenol Red	1.242	3.3E-3	1.242	3.3E-6

This stock is stored at room temperature in tightly stoppered bottles.

TABLE 6

STOCKS 6a (16.6X) AND 6b AND 6c (100X EACH)

Stock	Component	Concentration in Stock Solution		Concentration in Final Medium	
		g/l	*mol/l*	*mg/l*	*mol/l*
6a	Glutamine	14.62	1.0E-1	877.2	6.0E-3
6b	Sodium pyruvate	5.50	5.0E-2	55.0	5.0E-4
6c	Riboflavin	0.003764	1.0E-5	0.03764	1.0E-7

The original Stock 6 of medium F-12 (4), which contained four relatively reactive components, is no longer prepared as a single stock solution (9). Currently, glutamine, riboflavin, and sodium pyruvate are prepared as separate stocks and added individually when the final medium is made. Glucose is weighed out and added directly to the final medium. All three of these stocks should be stored frozen at −20° C if kept for an appreciable time. In addition, Stock 6c (riboflavin) must be protected from light.

TABLE 7

STOCK 7 (100X)

Component	Concentration in Stock Solution		Concentration in Final Medium	
	g/l	*mol/l*	*mg/l*	*mol/l*
Cysteine · HCl · H_2O	4.204	2.4E-2	42.04	2.4E-4

This stock must be discarded if it contains a precipitate. Deterioration of the solution can be minimized by keeping it tightly stoppered to reduce air oxidation. Stock 7 should be kept frozen at −20° C for storage of more than a few days.

TABLE 8

STOCK 8 (100X)

Component	Concentration in Stock Solution		Concentration in Final Medium	
	g/l	*mol/l*	*mg/l*	*mol/l*
Asparagine	1.501	1.0E-2	15.01	1.0E-4
Proline	3.453	3.0E-2	34.53	3.0E-4
Putrescine	0.01611	1.0E-4	0.1611	1.0E-6
Vitamin B_{12}	0.0407	3.0E-5	4.07	3.0E-7

This stock is stored at 4° C for up to 2 months or frozen at −20° C for longer periods.

TABLE 9

STOCK 9 (100X)

Component	Concentration in Stock Solution		Concentration in Final Medium	
	g/l	*mol/l*	*mg/l*	*mol/l*
Alanine	0.891	1.0E-2	8.91	1.0E-4
Aspartic acid	0.399	3.0E-3	3.99	3.0E-5
Glutamic acid	1.471	1.0E-2	14.71	1.0E-4
Glycine	0.751	1.0E-2	7.51	1.0E-4

This stock is prepared as follows: The aspartic and glutamic acids are added to slightly less than the final volume of water. One milliliter per liter of Phenol Red indicator solution (Stock 5) is added and 1.0 *N* sodium hydroxide is added with stirring just rapidly enough to keep the solution neutral (orange) as the aspartic and glutamic acids dissolve. When no solids remain and a stable orange colored solution is achieved the alanine and glycine are dissolved in the solution and the rest of the water is added to yield the final volume. Stock 9 is stored at 4° C for up to 2 months, or frozen at −20° C for longer periods.

TABLE 10

STOCK 10 (100X)

Component	Concentration in Stock Solution		Concentration in Final Medium	
	g/l	*mol/l*	*mg/l*	*mol/l*
Adenine	2.432	1.8E-2	24.32	1.8E-4
Inositol	1.802	1.0E-2	18.02	1.0E-4
Lipoic acid	0.02063	1.0E-4	0.2063	1.0E-6
Thymidine	0.07266	3.0E-4	0.7266	3.0E-6
$CuSO_4 \cdot 5H_2O$	0.000249	1.0E-6	0.00249	1.0E-8

This stock is prepared as follows: The adenine is added to 0.6 ml of 4 *N* NaOH, warmed to 40° C to dissolve resistant particles and then diluted with water. The lipoic acid is dissolved in a few drops of 1.0 *N* NaOH and then diluted and added to the stock solution. These two components dissolve readily when these procedures are followed but are nearly impossible to dissolve if added directly to the final solution. The other three components dissolve readily. Stock 10 is stored at 4° C for up to 2 months or frozen at −20° C for longer periods.

TABLE 11

STOCK 11 (1000X)

Component	Concentration in Stock Solution		Concentration in Final Medium	
	g/l	*mol/l*	*mg/l*	*mol/l*
$ZnSO_4 \cdot 7H_2O$	0.863	3.0E-3	0.863	3.0E-6

This stock is stored at room temperature in tightly stoppered bottles. *Stock 11 is normally not added to the final medium until immediately before use.*

TABLE 12

COMPONENTS ADDED DIRECTLY TO THE FINAL MEDIUM

Component	Concentration in Final Medium	
	mg/l	*mol/l*
Glucose	1081	6.0E-3
Sodium chloride	7599	1.3E-1
Sodium bicarbonate	1176	1.4E-2
Sodium acetate $\cdot 3H_2O$	500	3.7E-3
HEPES	6600	2.8E-2

Source of chemicals. All currently used biochemicals, including glucose, are obtained from Sigma Chemical Company, St. Louis, MO. All inorganic salts are from Fisher Scientific, Pittsburgh, PA. Phenol Red is from Sigma. The water is passed through a Millipore RO_4 reverse osmosis unit and then through a Millipore Milli-Q deionizing unit.

Preparation of final medium. In this laboratory MCDB 151 is generally prepared in 1-liter lots by adding the necessary stocks and chemicals to triple distilled water. *Stocks 4 and 11 are normally not added to the medium until just before it is to be used.*

The medium (minus Stocks 4 and 11) is then filtered through a type GSTF Millipore filter (0.22-μm pore size, detergent free). The medium is stored frozen at −20° C in volumes convenient for a single experiment (normally 200 to 500 ml).

TABLE 13

PREPRATION OF ONE LITER OF FINAL MEDIUM

Triple distilled water	800 ml
add with stirring:	
Stock 1	10 ml
Stock 2	10 ml
Stock 3	20 ml
Stock 5	1 ml
Stock 6a	60 ml
Stock 6b	10 ml
Stock 6c	10 ml
Stock 7	10 ml
Stock 8	10 ml
Stock 9	10 ml
Stock 10	10 ml
Glucose	1.081 g
Sodium chloride	7.599 g
Sodium acetate	0.5 g
HEPES	6.6 g
Adjust to pH 7.4 with 4.0 *N* NaOH and then add	
Sodium bicarbonate	1.176 g
Add triple distilled water to final volume of 989 ml	

When the medium is ready to be used, it is thawed by placing the bottle in a 37° C shaking water bath, and sterile Stocks 4 and 11 are added. Stocks 4 and 11 are sterilized by Millipore filtration or by autoclaving.

The amounts of stocks added per 100 ml are as follows:

Stock 4	1.0 ml
Stock 11	0.1 ml

All media were supplemented with 10 μg/ml HC (2.8×10^{-5} *M*) and whole or dialyzed serum at the levels indicated. A single lot of fetal bovine serum (Flow Laboratories Lot No. 40551248) was used for all experiments described in detail in this paper. However, similar results have also been obtained with other serum lots in more recent studies. Dialyzed fetal bovine serum protein (FBSP) was prepared by extensive dialysis against deionized water, lyophilization, and reconstitution in saline of weighed amounts of the dried powder, as previously described (10), except that the serum was not treated with EDTA unless indicated in the experiments. Pituitary extract (PE) was prepared as previously described (1).

The indirect immunofluorescence assays were performed by Dr. Bengt Westermark. Rabbit antiserum specific for human keratin (11,12) was obtained from Dr. Howard Green, Department of Biology, Massachusetts Institute of Technology. Fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG was obtained from Miles Research Products, Elkhart, IN. Cover slip-grown cultures were rinsed briefly with phosphate buffered saline (PBS) and fixed for 20 min at room temperature in a 2% solution of paraformaldehyde in PBS. The cover slips were rinsed with PBS and treated for 5 min at room temperature with 95% ethanol, after which they were rinsed again with PBS and incubated for 30 min at 37° C in the antikeratin serum diluted 1:20 with PBS. The cover slips were then rinsed with PBS and incubated for 30 min with FITC-conjugated goat antirabbit IgG diluted 1:10 with PBS. The cover slips were rinsed again with PBS and mounted in Elvanol. They were viewed and photographed with a Leitz Orthoplan microscope equipped with a mercury lamp and optics for epifluorescence.

RESULTS

Growth of HK with dialyzed serum. The following media, all supplemented with 10 μg/ml HC, 0.15 mg/ml each of PE 3A and 3B, and 5 mg/ml FBSP, were tested for their ability to support clonal growth of HK: medium 199, DME (low and high glucose), RPMI 1640, F-12, modified L-15, modified McCoy's 5-A, Waymouth medium MB 752/1, F-10, modified MEM, MCDB 104, MCDB 201, MCDB 401, and MCDB 411. Each dish was inoculated with 5×10^3 cells and the cultures were stained after 14 days. Colonies were present only on the plates with medium F-12. Medium 199, which supports excellent growth with whole serum, was ineffective with dialyzed serum.

Although the addition of pituitary extract fractions to medium 199 supplemented with 10 μg/ml HC and 20% (v/v) wFBS had been one of the major factors in obtaining growth of keratinocytes without a fibroblast feeder layer or medium conditioned by fibroblasts (1), experiments performed in medium F-12 supplemented with 10 μg/ml HC and 5 mg/ml FBSP revealed that PE fractions were of little or no benefit. The PE was therefore eliminated in subsequent experiments.

Development of medium MCDB 150. A series of steps to improve the growth of HK in medium

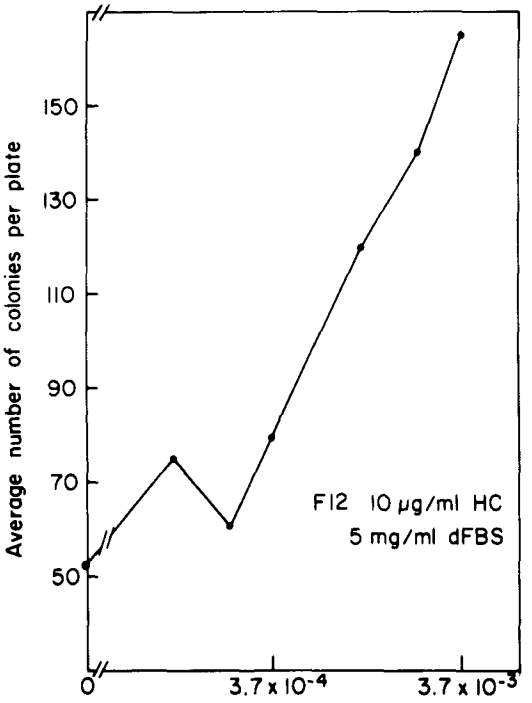

FIG. 1. Growth response of HK to sodium acetate. The indicated amounts of sodium acetate (0.2, 0.5, 1, 2, 5, or 10 times the level in medium 199, 3.7×10^{-4} *M*) were added to F-12 supplemented with 10 μg/ml HC and 5 mg/ml FBSP; 4.5×10^3 cells were inoculated into each dish and the dishes were incubated undisturbed for 14 days. The colonies were fixed, stained with Rhodanile Blue, and counted. Colony size was approximately equivalent on all of the plates. Each point represents the number of colonies per dish averaged from two dishes.

TABLE 14

CHANGES FROM THE COMPOSITION OF F-12 IN THE FORMULATION OF MCDB 150

Component	F-12	MCDB 150
L-Leucine	1.0E-4[a]	1.0E-3
L-Tryptophan	1.0E-5	3.0E-5
L-Valine	1.0E-4	3.0E-4
L-Serine	1.0E-4	6.0E-4
L-Glutamine	1.0E-3	6.0E-3
L-Cysteine	2.0E-4	1.2E-4
L-Aspartate	1.0E-4	3.0E-5
Folic acid	3.0E-6	1.8E-6
Vitamin B_{12}	1.0E-6	3.0E-7
Adenine	—	1.8E-4
Hypoxanthine	3.0E-5	—
Linoleic acid	3.0E-7	—
Glucose	1.0E-2	6.0E-3
Na acetate	—	3.7E-3

[a] An abbreviated exponential notation is used in which the letter "E" signifies "10 to the power." Thus, 1.0E-4 means 1.0×10^{-4} *M*.

F-12 with dialyzed serum was then undertaken. First, components present in medium 199 but not in medium F-12 were added individually to medium F-12 supplemented with 10 μg/ml HC and 5 mg/ml FBSP, at levels of 0.3 to 10 times those in medium 199. The compounds tested were ascorbic acid, ribose, ATP, calciferol, cholesterol, deoxyribose, glutathione, guanine, menadione, *p*-aminobenzoic acid, tocopherol, uracil, xanthine, retinyl acetate, and sodium acetate. Of these, ATP and sodium acetate were found to be stimulatory in the initial testing. The requirement for the addition of ATP was later eliminated by changes in the purine composition of the medium. Testing of sodium acetate over a wider range of concentrations revealed that the addition of 3.7×10^{-3} *M* to F-12 was optimal (Fig. 1). This concentration was included in the formulation of the first medium derived from F-12.

Next, the optimal concentration of each component in F-12 was determined by testing each at 0.1 to 10 times its normal level. The results of the clonal growth assays indicated that several changes in the quantitative composition of medium F-12 would improve clonal growth of HK under the test conditions (Table 14).

The new medium, which incorporated all of the changes that had been found to be beneficial, was designated MCDB 150, following the nomenclature scheme for media developed in this laboratory for different cell types (3). Changes that were made included a decrease in the levels of two amino acids (cysteine and aspartate) and an increase in the levels of five others (leucine, tryptophan, valine, serine, and glutamine). An increase in one of these (serine) had previously been reported by Liu and Karasek (13) to be beneficial for growth of HK. The response to increased

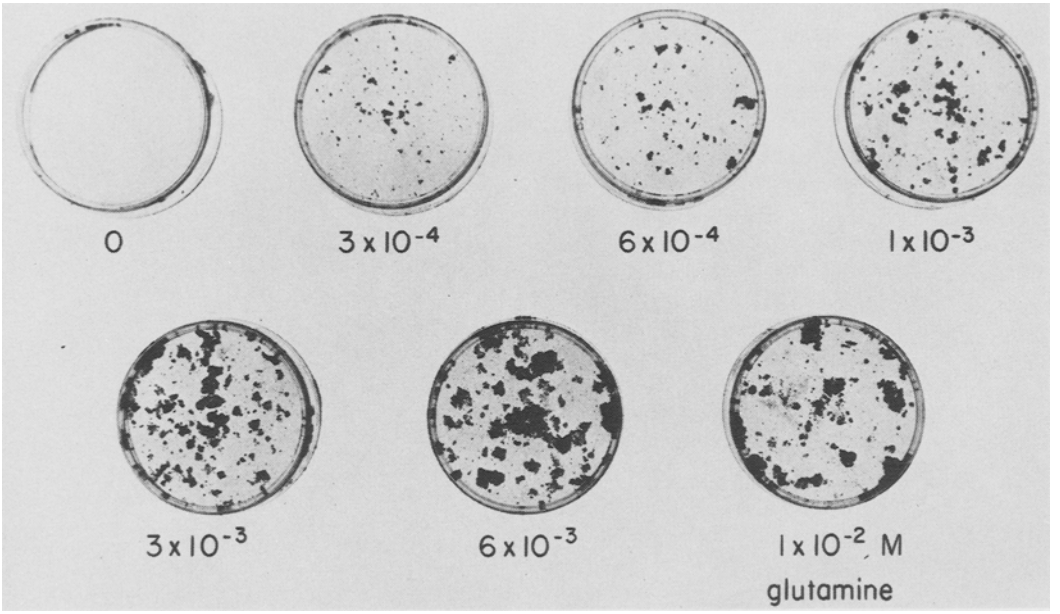

FIG. 2. Clonal growth of HK with increasing levels of glutamine. Concentrations of glutamine as indicated were added to F-12 prepared without glutamine and supplemented with 10 μg/ml HC and 5 mg/ml FBSP; 5×10^3 cells were inoculated per dish. Colonies were fixed and stained after 14 days.

levels of glutamine is illustrated in Fig. 2. The concentrations of folic acid, vitamin B_{12}, and glucose were all lowered. Linoleic acid was eliminated, and adenine at a much higher concentration replaced hypoxanthine. The response of HK to increased concentrations of adenine is shown in Fig. 3.

In F-12 with 10 μg/ml HC, no colonies were obtained with less than 3 mg/ml FBSP. However, in MCDB 150 with 10 μg/ml HC, colonies were detected with as little as 1 mg/ml FBSP. The specific adjustments that had been made in the small molecular weight components of the culture system thus satisfied some of the growth requirements that were previously dependent on a high concentration of FBSP.

Development of medium MCDB 151. After development of medium MCDB 150, a second full round of growth response titrations was performed. Medium MCDB 150 supplemented with 10 μg/ml HC and 2.5 mg/ml FBSP was used as the basal medium and each component was tested at 0, 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, and 10 times its level in MCDB 150. Modifications were again indicated, and the final composition of the resulting medium, MCDB 151, is shown in Table 15. Concentrations of four more amino acids were reduced (isoleucine, tyrosine, histidine, and lysine), as were the amounts of ferrous sulfate, sodium pyruvate, and potassium chloride. In addition, the levels of biotin and sodium phosphate were raised and the concentration of calcium was reduced substantially (from 3×10^{-4} *M* to 3×10^{-5} *M*). The response of HK to calcium is shown in Fig. 4. In medium MCDB 151 with 10 μg/ml HC, optimal growth was obtained with only 1 mg/ml FBSP, and some colony formation was observed with as little as 0.5 mg/ml FBSP.

Primary growth. A comparison of MCDB 151 with MCDB 105, which was developed in this laboratory for the growth of diploid human fibroblasts with low amounts of serum protein, revealed numerous differences (Table 15). Such discrepancies in the growth requirements of the two cell types could potentially mediate a selective advantage for growth of a given cell type. Primary suspensions of disaggregated foreskin cells (1) were inoculated into MCDB 151 supplemented

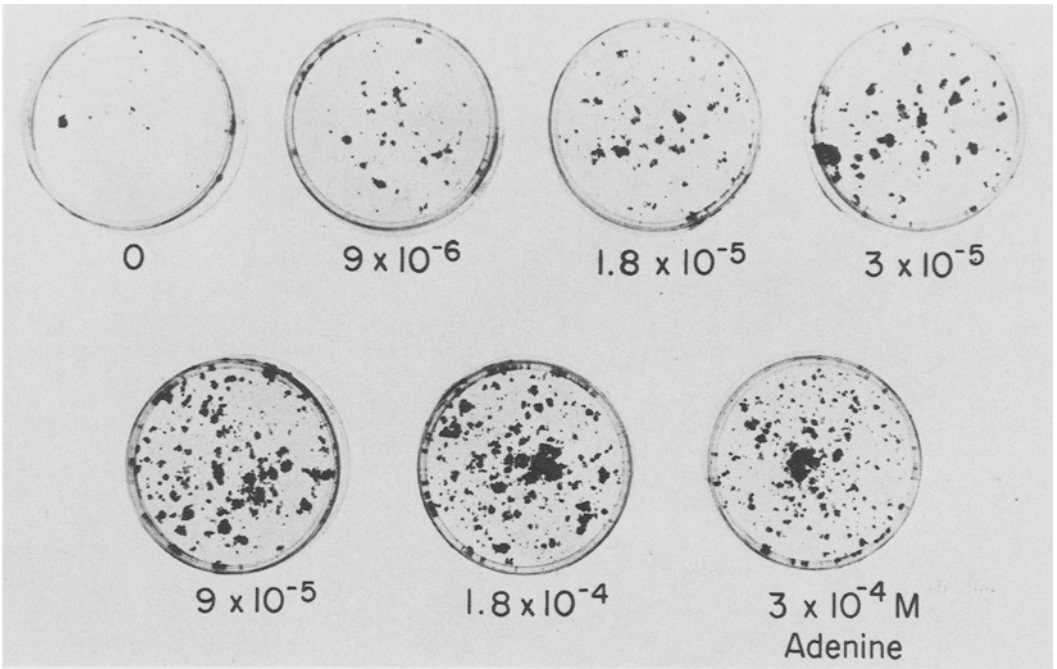

FIG. 3. Clonal growth of HK with increasing levels of adenine. 5×10^3 Cells were plated per 35-mm dish in F-12 prepared without hypoxanthine and supplemented with 10 μg/ml HC and 5.0 mg/ml FBSP. Adenine was added at the indicated concentrations. The cultures were incubated undisturbed until they were fixed and stained on Day 14. Growth was compared visually and 1.8×10^{-4} *M* adenine was judged to be optimal.

TABLE 15

COMPARISON OF MEDIA FOR HUMAN DIPLOID KERATINOCYTES AND FIBROBLASTS

Name of Medium	MCDB 151	MCDB 105	F-12
Essential amino acids			
Arginine	1.0E-3[a]	1.0E-3	1.0E-3
Cysteine	2.4E-4	5.0E-5	2.0E-4
Half-cystine	—	—	—
Glutamine	6.0E-3	2.5E-3	1.0E-3
Histidine	8.0E-5	1.0E-4	1.0E-4
Isoleucine	1.5E-5	3.0E-5	3.0E-5
Leucine	5.0E-4	1.0E-4	1.0E-4
Lysine	1.0E-4	2.0E-4	2.0E-4
Methionine	3.0E-5	3.0E-5	3.0E-5
Phenylalanine	3.0E-5	3.0E-5	3.0E-5
Threonine	1.0E-4	1.0E-4	1.0E-4
Tryptophan	1.5E-5	1.0E-5	1.0E-5
Tyrosine	1.5E-5	3.0E-5	3.0E-5
Valine	3.0E-4	1.0E-4	1.0E-4
Nonessential amino acids			
Alanine	1.0E-4	1.0E-4	1.0E-4
Asparagine	1.0E-4	1.0E-4	1.0E-4
Aspartate	3.0E-5	1.0E-4	1.0E-4
Glutamate	1.0E-4	1.0E-4	1.0E-4
Glycine	1.0E-4	1.0E-4	1.0E-4
Proline	3.0E-4	3.0E-4	3.0E-4
Serine	6.0E-4	1.0E-4	1.0E-4
Vitamins			
Biotin	6.0E-8	3.0E-8	3.0E-8
Folate	1.8E-6	—	3.0E-6
Folinate	—	1.0E-9	—
Lipoate	1.0E-6	1.0E-8	1.0E-6
Niacinamide	3.0E-7	5.0E-5	3.0E-7
Pantothenate	1.0E-6	1.0E-6	1.0E-6
Pyridoxine	3.0E-7	3.0E-7	3.0E-7
Riboflavin	1.0E-7	3.0E-7	1.0E-7
Thiamin	1.0E-6	1.0E-6	1.0E-6
Vitamin B_{12}	3.0E-7	1.0E-7	1.0E-6
Purines and pyrimidines			
Adenine	1.8E-4	1.0E-5	—
Hypoxanthine	—	—	3.0E-5
Thymidine	3.0E-6	3.0E-7	3.0E-6
Other organic compounds			
Acetate	3.7E-3	—	—
Choline	1.0E-4	1.0E-4	1.0E-4
Glucose	6.0E-3	4.0E-3	1.0E-2
i-Inositol	1.0E-4	1.0E-4	1.0E-4
Linoleate	—	1.0E-8	3.0E-7
Putrescine	1.0E-6	1.0E-9	1.0E-6
Pyruvate	5.0E-4	1.0E-3	1.0E-3
Major inorganic ions			
Calcium	3.0E-5	1.0E-3	3.0E-4
Magnesium	6.0E-4	1.0E-3	6.0E-4
Potassium	1.5E-3	3.0E-3	3.0E-3
Sodium	1.5E-1	1.3E-1	1.5E-1
Chloride	1.3E-1	1.2E-1	1.4E-1
Phosphate	2.0E-3	3.0E-3	1.0E-3
Sulfate	4.5E-6	1.0E-3	6.0E-6
Trace elements			
Copper	1.0E-8	1.0E-9	1.0E-8
Iron	1.5E-6	5.0E-6	3.0E-6
Manganese	—	1.0E-9	—
Molybdenum	—	7.0E-9	—
Nickel	—	5.0E-10	—

TABLE 15 (continued)

Name of Medium	MCDB 151	MCDB 105	F-12
Selenium	—	3.0E-8	—
Silicon	—	5.0E-7	—
Tin	—	5.0E-10	—
Vanadium	—	5.0E-9	—
Zinc	3.0E-6	5.0E-7	3.0E-6
Buffers and indicators			
Bicarbonate	1.4E-2	—	1.4E-2
Carbon dioxide	5%	2%	5%
HEPES	2.8E-2	3.0E-2	—
Phenol Red	3.3E-6	3.3E-6	3.3E-6
Supplementation for clonal growth			
Hydrocortisone			
Hydrocortisone	2.7E-5	—	—
FBSP (μg/ml)	1000	500-1000	—[b]

[a] An abbreviated exponential notation is used in which the letter "E" signifies "10 to the power." Thus, 1.0E-3 means 1.0×10^{-3} *M*.

[b] F-12 originally developed for clonal growth of Chinese hamster cell lines without protein supplementation (4).

with 10 μg/ml HC and 1 mg/ml FBSP, or into MCDB 105 supplemented with 1 mg/ml FBSP. No irradiated 3T3 cells were used. The cells were fed every 3 days and the type and amount of growth after 8 days was examined. Figure 5 illustrates the selective growth that occurs under such conditions. In MCDB 105, a few keratinocyte colonies are seen surrounded by a lawn of fibroblasts. In MCDB 151, a few fibroblasts are apparent, but the predominant growth is that of keratinocytes. The two cell types are rather easily identifiable by morphology, but the stain used, Rhodanile Blue, more clearly identifies the cell types on the basis of differential staining (keratinocytes retain the red component of the stain, whereas fibroblasts retain only the blue component).

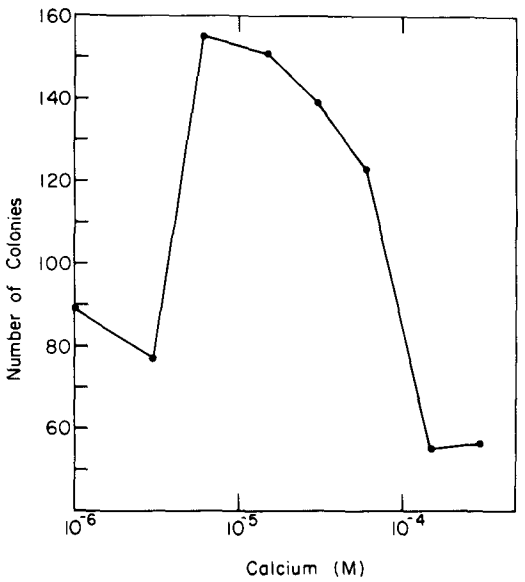

FIG. 4. Growth response of HK to calcium in MCDB 150. 5×10^3 Cells per dish were inoculated into MCDB 150 prepared without Ca^{2+} and supplemented with 10 μg/ml HC and 2.5 mg/ml FBSP. Calcium chloride was added at the indicated concentrations. The average number of colonies per dish was determined by counting fixed and stained colonies after 14 days of growth. Four plates were counted per point.

Components mediating selective growth. One of the major differences between the system used to culture human fibroblasts and that used to culture keratinocytes is the high level of hydrocortisone required by the keratinocytes. Raising the level of HC from 0.4 to 10 μg/ml was the initial change that permitted colony formation by HK in unconditioned medium (1). This high level of HC is also necessary for the optimal growth of HK in MCDB 151 with 1 mg/ml FBSP. However, although it has been reported that HC inhibits the growth of human foreskin fibroblasts (14), the inhibition by HC alone is not sufficient to prevent growth of fibroblasts in MCDB 151 since numerous fibroblasts appear when suspensions of foreskin cells are inoculated into MCDB 150 or F-12 with the same amount of HC.

Another potential difference mediating selection is the level of adenine. Adenine is not a component of F-12, but since the replacement of hypoxanthine by adenine was found to be beneficial when MCDB 105 was developed for fibroblasts (5) the same experiment was done with the keratinocytes. Not only was the replacement of hypoxanthine by adenine beneficial, but raising the level of adenine to 1.8×10^{-4} *M* was one of the

most important changes in the development of MCDB 150 and subsequent reduction in the amount of serum required. This level of adenine is apparently quite toxic to fibroblasts, since the inoculation of primary foreskin cells into MCDB 151 with 1.8×10^{-5} *M* adenine rather than 1.8×10^{-4} *M* results in the growth of numerous fibroblasts.

The difference in the levels of calcium in MCDB 105 and MCDB 151 is striking and quite interesting with regard to the postulated role of calcium in control of cellular proliferation and differentiation. The low level of calcium in MCDB 151, 3×10^{-5} *M*, supports optimal growth of HK, but the morphology of the colonies is radically altered. Monolayers composed of rather loosely packed cells develop, rather than the stratified colonies of closely packed cells that form in media with a higher level of calcium (Fig. 6). This effect is readily reversible. Monolayer colonies become stratified in less than 24 hr after the level of calcium is raised to a higher level. Similar results have been reported for mouse epidermal keratinocytes (15). Although the cells remain as a monolayer with the lower level of calcium, keratin is still apparent as demonstrated by staining with fluorescent antibodies against keratin (Fig. 7).

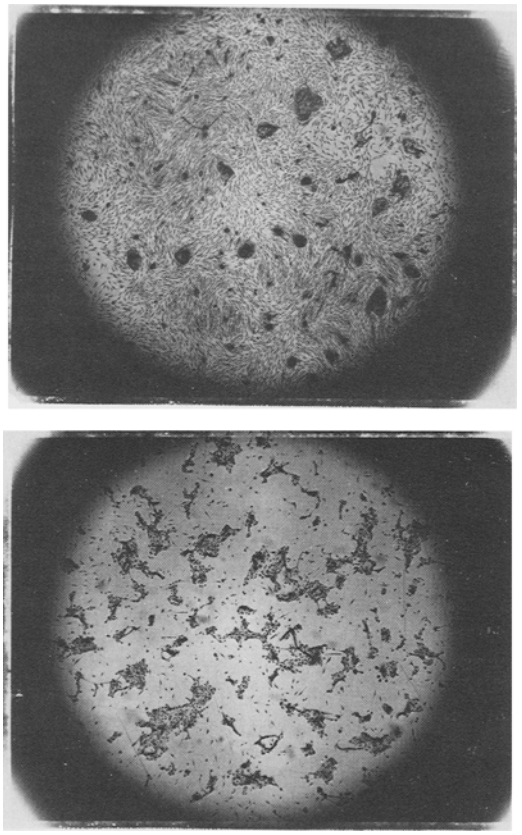

FIG. 5. Selective growth of fibroblasts or keratinocytes in MCDB 105 or MCDB 151. 1×10^5 Cells from a primary suspension of disaggregated foreskin cells were plated into 35-mm dishes with MCDB 105 supplemented with 1 mg/ml FBSP or MCDB 151 supplemented with 10 μg/ml HC and 1 mg/ml FBSP. The cells were fed every 3 days until they were fixed and stained with Rhodanile Blue on Day 8. Fibroblasts predominate in MCDB 105 (*top*), whereas keratinocytes prosper in MCDB 151 with only a few fibroblasts evident (*bottom*).

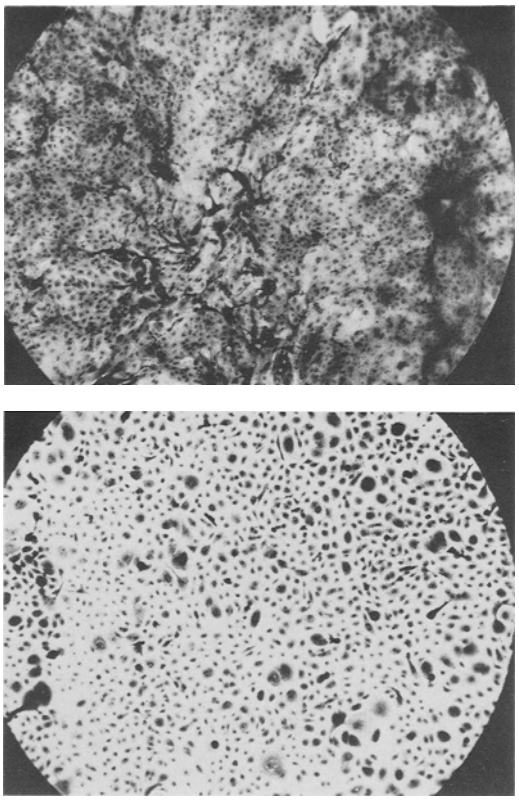

FIG. 6. Morphology of HK cultured in MCDB 151 with 3×10^{-4} or 3×10^{-5} *M* calcium. Cells were inoculated into MCDB 151, with 3×10^{-4} (*top*) or 3×10^{-5} *M* (*bottom*) calcium, supplemented with 10 μg/ml HC and 1.0 mg/ml FBSP. Colonies were fixed and stained with Rhodanile Blue on Day 14. ×10.

In view of these results, the poor growth of HK in MCDB 105 could partially be due to the high level of calcium (1×10^{-3} *M*) in that medium. A clonal growth assay of HK in MCDB 105 supplemented with 10 μg/ml HC and 1 mg/ml FBSP, and with the calcium concentration varied between 10^{-6} and 10^{-3} *M*, showed indeed that while no clonal growth occurred in the presence of the

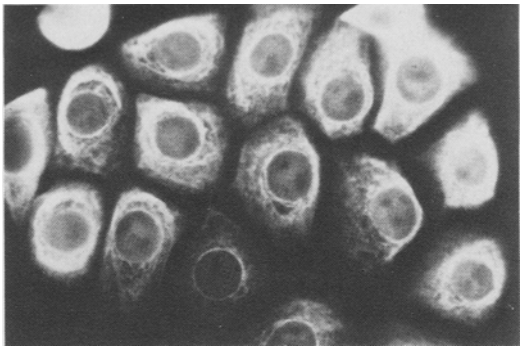

FIG. 7. Fluorescent antibody labeling of keratin in HK growing as a monolayer in MCDB 151. Cells were cultured in MCDB 151 supplemented with 10 μg/ml HC and 1.0 mg/ml FBSP for 5 days, then fixed and labeled with rabbit antihuman keratin followed by fluorescent goat antirabbit immunoglobulin.

higher levels of calcium, colonies did form with less than 10^{-4} *M* calcium (Fig. 8).

During the development of MCDB 151, a series of experiments were performed to determine whether a different type of selection against the growth of fibroblasts could be achieved. It has been reported that the substitution of D-valine for L-valine in culture media can selectively inhibit the growth of fibroblasts while allowing the proliferation of certain types of epithelial cells that possess a D-amino acid oxidase (16). Keratinocytes, however, apparently do not have this ability, since no clonal growth occurred in medium F-12 with D-valine substituted for L-valine, except above the levels at which the L-valine contaminant in the D-valine (3%) would have been expected to support growth (Fig. 9). Keratinocytes therefore appear to be unamenable for use in such a selection scheme.

Other factors. The effect of hormones, vitamins, and other "growth factors" on clonal growth of HK were studied previously with conditioned medium and with medium 199 supplemented with PE and whole serum (1). Several were tested again under the more defined conditions described in this paper, since their effects on HK might be apparent only in the presence of limited amounts of serum protein. Epidermal growth factor and fibroblast growth factor, from 0.1 to 10 mg/ml, were again found to be without effect. Insulin added at concentrations as high as 10 μg/ml is also ineffectual. Vitamin B_{12}, not present in medium 199, was previously found to be somewhat stimulatory when added at 1×10^{-5} *M* to medium 199 supplemented with 10 μg/ml HC and 20% (v/v) wFBS. The level of vitamin B_{12} present in F-12 was found to be somewhat toxic with low amounts of dialyzed serum used and was lowered to 3×10^{-7} *M*.

Green has reported that the addition of agents that raise the level of intracellular cAMP to cultures of keratinocytes stimulates their growth, even in the absence of fibroblasts (17). However, no such stimulation could be observed upon the addition of dibutyryl cAMP (4×10^{-5} to 4×10^{-3} *M*), theophylline (4×10^{-5} to 4×10^{-3} *M*), or cholera toxin (1.2×10^{-10} *M* to 1.2×10^{-8} *M*) to MCDB 151 with 10 μg/ml HC and 1.0 mg/ml FBSP. The results of the addition of cholera toxin to HK cultures are shown in Fig. 10. The morphology of the colonies appears somewhat altered in the presence of cholera toxin. The colonies are more diffuse, remaining flattened with slightly separated cells.

DISCUSSION

Two important conclusions can be drawn from the results presented in this paper: (a) the growth requirements of human epidermal keratinocytes differ sufficiently from those of human fibroblast-like cells so that it is possible to develop media that selectively favor growth of either cell type;

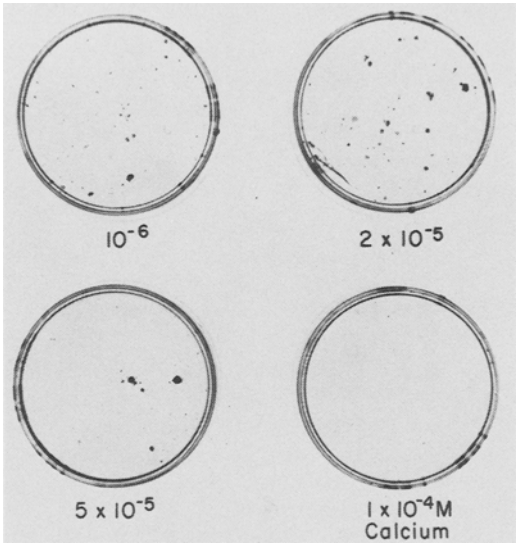

FIG. 8. Clonal growth of HK in MCDB 105 with altered levels of calcium. 3×10^3 Cells were inoculated into MCDB 105 prepared without $CaCl_2 \cdot 2H_2O$ and supplemented with 10 μg/ml HC and 1.0 mg/ml FBSP. $CaCl_2 \cdot 2H_2O$ was added at the indicated levels. The plates were fixed and stained on Day 14.

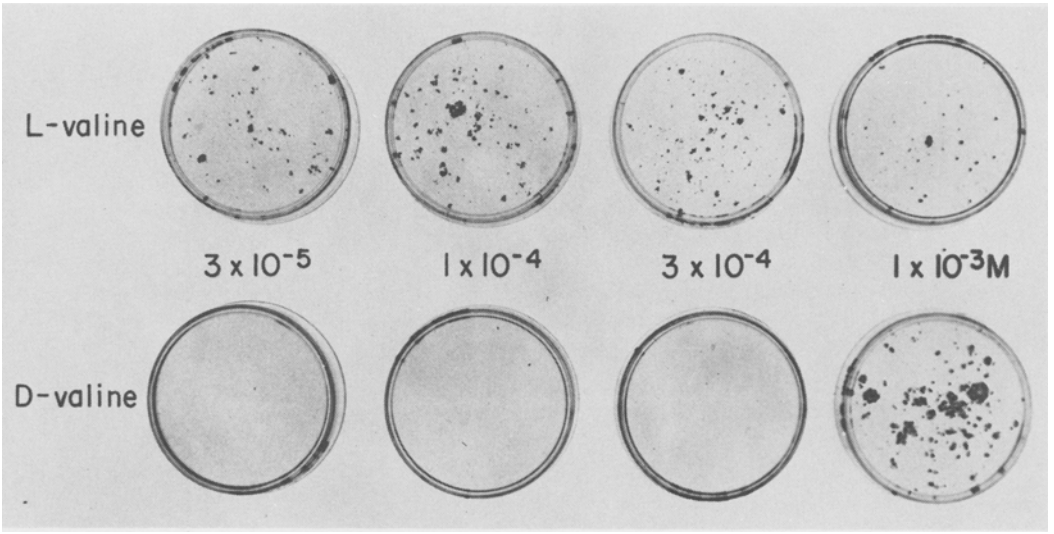

FIG. 9. Clonal growth of keratinocytes in media with D- or L-valine. Medium F-12 was prepared without valine and supplemented with 10 μg/ml HC and 5 mg/ml FBSP. D- or L-valine was added at the levels indicated and the dishes were inoculated with 4×10^3 cells each. Colonies were fixed and stained after 14 days.

and (b) there is nothing vastly or mystifyingly different about the growth requirements of keratinocytes. It is obviously too soon to generalize, but the results presented here strongly suggest that similar conclusions will also be valid for other types of epithelial cells, which in the past have been extremely difficult to grow in culture.

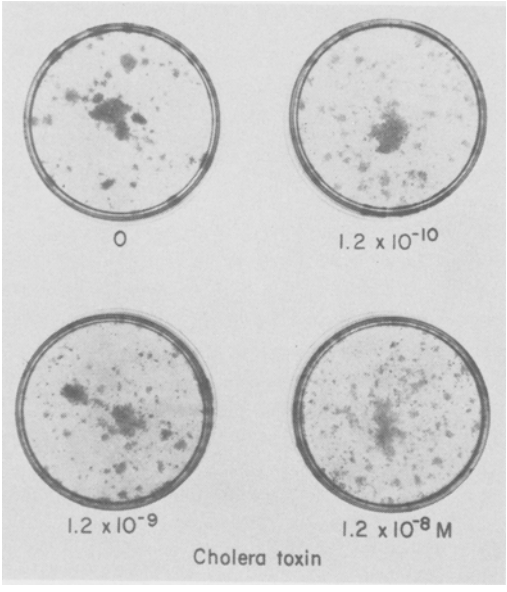

FIG. 10. Growth of keratinocytes with cholera toxin. 3×10^3 Cells were inoculated into MCDB 151 supplemented with 10 μg/ml HC and 1.0 mg/ml FBSP. Cholera toxin was added at concentrations from 1.2×10^{-10} *M* to 1.2×10^{-8} *M*. Plates were fixed and stained on Day 14. No obvious growth stimulation results from the addition of cholera toxin under these conditions.

The results presented in this paper strongly reinforce the concept that each type of cell, even within the same species, has a unique set of quantitative growth requirements that must be satisfied in order to obtain satisfactory multiplication with minimal amounts of serum proteins (18). There is nothing qualitatively unique about the set of culture conditions that has been developed for clonal growth of human epidermal keratinocytes without a feeder layer or conditioned medium and with relatively small amounts of dialyzed serum protein as the only undefined supplement. Every component of the culture system has been employed many times before for growth of other types of cells. The unique requirements for clonal growth of HK without large amounts of whole serum or interaction with other cell types are thus entirely quantitative in nature.

It is interesting to note that many of the experimental approaches that were utilized in the development of a medium specifically for these epithelial cells were originally developed through the use of fibroblastic cells (3,7,8). There is no reason to believe that diligent application of techniques such as progressive quantitative optimization of

the culture medium and other aspects of the culture environment at progressively lower concentrations of dialyzed serum protein cannot yield similar results for other types of epithelial cells that are currently very difficult to grow. An important first step in the current investigation was utilization of the feeder-layer technique, which was pioneered by Puck and Marcus (19) and first applied to growth of human epidermal keratinocytes by Rheinwald and Green (6). Use of the feeder layer made possible an adequate level of growth so that a systematic process of optimization and elimination of undefined components (starting with the feeder layer) could then be undertaken.

It is not possible to generalize about the growth requirements of epithelial cells on the basis of experiments with a single cell type. However, it will be interesting to determine whether other epithelial cells that are presently difficult to culture, and especially those of human origin, will grow better in MCDB 151 than in other media, which generally have been developed primarily for fibroblastic cells (20,21).

Clonal growth of human epidermal keratinocytes in a defined medium with the addition of only small amounts of undefined supplements opens the way for studies seeking to identify the remaining growth-promoting substances from dialyzed serum, as well as for studies of environmental signals that influence their growth and differentiation. The effect of Ca^{2+} concentration on keratinocyte growth and differentiation clearly deserves further study, for example. Already, we have observed a pattern very different from that in fibroblasts, and we have been able to dissociate one aspect of differentiation (stratification) from growth through manipulation of the Ca^{2+} concentration. Further studies will determine whether the loss of stratification that occurs with low levels of calcium correlates with the loss of other differentiated properties (e.g., cross-linked envelopes, loss of nuclei, etcetera), or if the cells still follow the normal pattern of terminal differentiation in a monolayer, as suggested by the continued presence of keratin.

The amount of dialyzed serum protein needed for optimal clonal growth of normal human keratinocytes has now been reduced to a value equivalent to that required for optimal clonal growth of normal human fibroblasts in MCDB 105, although suboptimal colony formation can be obtained with lower levels of FBSP with the fibroblasts than with the keratinocytes. Attempts to characterize the remaining serum macromolecules that are needed by the keratinocytes are still in early stages. It will be most interesting to see whether there exist any qualitatively unique growth factors for the keratinocytes or whether their macromolecular requirements will turn out to be similar to those of fibroblasts, which are also still incompletely characterized (5).

The role of pituitary extract in growth of human epidermal keratinocytes is not yet fully resolved. Pituitary extract was essential for growth of keratinocytes in medium 199 (1) but was found not to be required in medium F-12 with relatively large amounts of FBSP and was not used during the development of medium MCDB 151. However, recent experiments performed with MCDB 151 have shown that pituitary extract will partially (but not fully) replace the FBSP requirement for keratinocyte growth. The lowest total protein concentration that will support growth of keratinocytes is achieved with a mixture of pituitary extract and FBSP.

The availability of two very different types of normal human diploid cells that can now be grown in synthetic media supplemented with limited amounts of dialyzed serum proteins should put an end to the dubious practice of attempting to generalize to all types of cells from the requirements of fibroblasts. The diversity of growth responses reported in this paper makes it adequately clear that such generalizations are not safe, and demonstrates that conditions that are optimal for one type of cell may actually inhibit the multiplication of another. It is reasonable to assume that other types of normal human cells whose growth requirements have not yet been explored in detail are likely to have quantitative (and perhaps also qualitative) growth requirements that are very different than those of fibroblasts or keratinocytes. Such requirements need to be studied not only for other epithelial cells, but also for mesodermal cells that are neither fibroblastic or epithelial in nature.

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