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Rapid clonal growth and serial passage of human diploid fibroblasts in a lipid-enriched synthetic medium supplemented with epidermal growth factor, insulin, and dexamethasone

(liposomes/phospholipids/cholesterol/prostaglandins/serum-free medium)

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ABSTRACT **A serum-free, hormone-supplemented medium, enriched with a mixture of lipids, has been developed that supports rapid clonal growth of human diploid fetal lung fibroblasts (Flow 2000, WI-38, MRC-5, and IMR-90) and of low-passage human foreskin fibroblasts. The medium, which contains less than 1 μg of total protein per ml, also supports serial passage of Flow 2000 cells under totally serum-free conditions. It provides lipid at a total of 10 μg/ml as a liposome prepared from a mixture of soybean lecithin, cholesterol, sphingomyelin, vitamin E, and vitamin E acetate. The soybean lecithin, which contains a variety of naturally occurring phospholipids, can be replaced with a mixture of highly purified phospholipids. Except for possible contaminants in the substances used in its preparation, the serum-free medium is fully defined chemically. It consists of an optimized basal nutrient medium, MCDB 110, supplemented with insulin, epidermal growth factor, dexamethasone, prostaglandins E_1 and $F_{2\alpha}$, phospho*enol*pyruvate, dithiothreitol, glutathione, and the lipids listed above.**

Rapid clonal growth of normal human diploid cells without serum or other undefined additives has not previously been achieved. In our laboratory, qualitative and quantitative optimization of culture media and improved culture methods have greatly reduced the amount of serum needed for clonal growth of normal human fibroblasts (1–3) and keratinocytes (4). Sato and coworkers have obtained rapid multiplication of many cell types in conventional media by replacing serum with hormones, growth factors, carrier proteins, and additional nutrients (5–7).

Neither of these two approaches alone is fully satisfactory for human diploid fibroblasts (HDF). However, a combination of optimized media plus hormones and growth factors supports significant multiplication of HDF (8–11) and normal human glial cells (12). This communication describes additional supplementation to yield a "defined" medium in which the rate of clonal growth of HDF approaches that obtained with serum.

MATERIALS AND METHODS

Media and Solutions. Medium MCDB 110 is the same as MCDB 104 (1) except for the following changes: linoleic acid is deleted; glycine is increased from 0.1 to 0.3 mM; KCl is increased from 3.0 to 5.0 mM; Hepes is reduced from 50 to 30 mM; NaCl is increased from 100 to 112 mM; and NaOH added to bring the pH in air at room temperature to 7.65 is reduced from ca. 26 mM to ca. 20 mM. MCDB 110 is used with 2% CO_2 rather than 5%. The molar composition of MCDB 110 is given in Table 1. MCDB 108 (10) is MCDB 110 plus 1.0×10^{-8} M linoleic acid.

Solution A contains 30 mM Hepes/NaOH (pH 7.6), 10 mM glucose, 3 mM KCl, 130 mM NaCl, 1.0 mM Na_2HPO_4 and 3.3 μM phenol red.

Supplement. Three multicomponent supplements (Table 2) are added to MCDB 110 for serum-free clonal growth of HDF. The water-soluble components of supplement A (dithiothreitol, glutathione, phospho*enol*pyruvate) are dissolved in solution A at 100× final concentration, sterilized by filtration, and stored frozen at −20°C. The two prostaglandins are dissolved separately in absolute ethanol each at 20,000× final concentration and stored at −20°C. Supplement A (100×) is prepared by adding 5 μl of each prostaglandin stock per ml of the aqueous solution. The prostaglandins are relatively unstable in aqueous solution. Hence only enough supplement A for immediate use should be prepared. Supplement A_0, used in early experiments, consists of supplement A plus 1 μM dibutyryl cyclic GMP and 5 nM dibutyryl cyclic AMP.

Supplement B is added to the medium as a liposome. Its components are dissolved individually in 2:1 (vol/vol) chloroform/methanol at 1000× the final concentration, mixed together in a test tube, and evaporated to dryness under a stream of nitrogen. The resulting lipid mixture is suspended in solution A (25°C) at 50× final concentration, equilibrated for 15 min, stirred briefly in a Vortex mixer, and then sonicated under N_2 at 25°C with a Fisher model 300 sonicator at 30% of maximum power with a 4-mm-diameter probe. Sonication is done for periods of 3 min, with alternating 1-min rest periods to aid in maintaining 25°C temperature, for a total time period of twice the clearing time of the suspension (ca. 30 min total sonication). The liposome preparation is then sterilized by passage through a 0.22-μm-pore-diameter Nuclepore filter (other types of filters may not yield equivalent results). Preparations that do not pass through filters readily are considered incompletely sonicated and are discarded. Supplement B should always be prepared within 24 hr before it is used in an experiment.

Supplement P-4 is identical to supplement B except that the commerical grade lecithin at 6 μg/ml is replaced by highly purified L-α-phosphatidylcholine (Sigma P 6263), L-α-phosphatidylethanolamine (Sigma P 4513), L-α-phosphatidylinositol (Sigma P 0639), and dipalmitoyl L-α-phosphatidic acid (sodium salt) (Sigma P 4013) at 1.5 μg/ml each. Supplement P-2 has highly purified phosphatidylcholine and phosphatidylethano-

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Abbreviations: EGF, epidermal growth factor; FBSP, fetal bovine serum protein (dialyzed, lyophilized, and reconstituted); HDF, human diploid fibroblasts.

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Table 1. Composition of medium MCDB 110*

Amino acids		Other organic compounds	
L-Alanine	1.0 E-4	Adenine	1.0 E-5
L-Arginine·HCl	1.0 E-3	Choline chloride	1.0 E-4
L-Asparagine·H_2O	1.0 E-4	D-Glucose	4.0 E-3
L-Aspartic acid	1.0 E-4	*i*-Inositol	1.0 E-4
L-Cysteine·HCl·H_2O	5.0 E-5	Putrescine·2HCl	1.0 E-9
L-Glutamic acid	1.0 E-4	Sodium pyruvate	1.0 E-3
L-Glutamine	2.5 E-3	Thymidine	3.0 E-7
Glycine	3.0 E-4	Major inorganic salts	
L-Histidine·HCl·H_2O	1.0 E-4	$CaCl_2 \cdot 2H_2O$	1.0 E-3
L-Isoleucine	3.0 E-5	KCl	5.0 E-3
L-Leucine	1.0 E-4	$MgSO_4 \cdot 7H_2O$	1.0 E-3
L-Lysine·HCl	2.0 E-4	NaCl	1.1 E-1
L-Methionine	3.0 E-5	$Na_2HPO_4 \cdot 7H_2O$	3.0 E-3
L-Phenylalanine	3.0 E-5	Trace elements	
L-Proline	3.0 E-4	$CuSO_4 \cdot 5H_2O$	1.0 E-9
L-Serine	1.0 E-4	$FeSO_4 \cdot 7H_2O$	5.0 E-6
L-Threonine	1.0 E-4	H_2SeO_3	3.0 E-8
L-Tryptophan	1.0 E-5	$MnSO_4 \cdot 5H_2O$	1.0 E-9
L-Tyrosine	3.0 E-5	$Na_2SiO_3 \cdot 9H_2O$	5.0 E-7
L-Valine	1.0 E-4	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$†	1.0 E-9
Vitamins		NH_4VO_3	5.0 E-9
d-Biotin	3.0 E-8	$NiCl_2 \cdot 6H_2O$	5.0 E-10
Folinic acid	1.0 E-9	$SnCl_2 \cdot 2H_2O$	5.0 E-10
DL-α-Lipoic Acid	1.0 E-8	$ZnSO_4 \cdot 7H_2O$	5.0 E-7
Niacinamide	5.0 E-5	Buffers and indicators	
D-Pantothenate·½Ca†	1.0 E-6	Hepes	3.0 E-2
Pyridoxine·HCl	3.0 E-7	NaOH	2.0 E-2
Riboflavin	3.0 E-7	Phenol red	3.3 E-6
Thiamin·HCl	1.0 E-6	pH in air, 22°C	7.65‡
Vitamin B-12	1.0 E-7	Osmolality (mOsm/kg)	285 ± 5

* The alphabetical listings in this table do not reflect the sequences and groupings used to prepare MCDB 110, which are essentially as described for MCDB 104 (1). Concentrations are given in moles per liter, expressed in computer-style exponential notation (e.g., 1.0 E-4 means 1.0×10^{-4} M). Water of hydration refers to the form in which the component was supplied.

† Molar concentrations are given for the component of interest (pantothenic acid, molybdenum) rather than for the molecular form in which it is supplied.

‡ Final pH at 37°C and equilibrated with 2% CO_2 is 7.25.

lamine at 3 μg/ml each. Supplement P-1 has highly purified phosphatidylcholine at 6 μg/ml.

Supplement C is prepared as follows: (*i*) dissolve dexamethasone in absolute ethanol at 2 mg/ml; (*ii*) dissolve insulin in 12 mM HCl at a concentration of 9.5 mg/84 ml; (*iii*) add 1.0 ml of the dexamethasone solution per 84 ml of the insulin solution, sterilize by filtration, and store frozen; (*iv*) reconstitute sterile lyophilized EGF in sterile solution A at 20 μg/ml and store frozen; (*v*) mix 8.5 ml of the insulin/dexamethasone solution with 1.5 ml of the EGF solution to prepare supplement C (100×). Supplement C can be stored frozen at −20°C in small aliquots, but it should not be repeatedly frozen and thawed. Supplement C_0, used in early experiments, contains insulin at 1.0 μg/ml, EGF at 60 ng/ml, and 0.5 μM dexamethasone. Its preparation is the same, except for appropriate increases in concentrations of the stock solutions.

EGF that migrates as a single band in sodium dodecyl sulfate/acrylamide gel electrophoresis is prepared in our laboratory by the method of Savage and Cohen (13). Commerical EGF (Collaborative Research, Waltham, MA:) can also be used. Whole fetal bovine serum is purchased from Flow Laboratories (McLean, VA). Dialyzed fetal bovine serum protein (FBSP) is prepared as described (14) except that EDTA is not added prior to dialysis.

Cells. Flow 2000 human fetal lung fibroblasts are purchased from Flow Laboratories (McLean, VA) at passage 8–9, grown to passage 11–12 in MCDB 108 plus 10% whole fetal bovine serum, and then frozen and stored under liquid nitrogen in 70% MCDB 108, 20% whole fetal bovine serum, and 10% dimethyl sulfoxide (vol/vol). The handling of WI-38 (ATCC-CCL 75), MRC-5 (ATCC-CCL 171), and IMR-90 (Institute for Medical Research, Camden, NJ) human diploid lung fibroblasts and of low-passage human diploid foreskin fibroblasts (grown in this laboratory from primary cultures) is essentially the same as for the Flow 2000 cells.

Table 2. Supplements used with MCDB 110 to replace serum

	Source*	Conc.† μg/ml	Conc.† M
Supplement A			
Dithiothreitol	Sigma D 0632	1.0	6.5 E-6
Glutathione (reduced)	Sigma G 4251	0.2	6.5 E-7
Phospho*enol*pyruvate	Sigma P 7127	2.1	1.0 E-5
Prostaglandin E_1	Sigma P 5515	0.009	2.5 E-8
Prostaglandin $F_{2\alpha}$	Sigma P 5890	0.71	2.0 E-6
Supplement B			
Cholesterol	Sigma Ch-S	3.0	7.6 E-6
Soybean lecithin (commercial grade)	Sigma P 5638	6.0	—
Sphingomyelin	Supelco 4-6009	1.0	—
Vitamin E	U.S.B. 22410	0.06	1.4 E-7
Vitamin E acetate	U.S.B. 22425	0.2	4.2 E-7
Supplement C			
Dexamethasone	Sigma D 1756	0.2	5.0 E-7
Epidermal growth factor	‡	0.03	5.0 E-9
Insulin	Sigma I 5500	0.95	1.6 E-7

* Because many of the components are available in a variety of forms and degrees of purity, catalog numbers are given for more complete identification. U.S.B., U.S. Biochemical.

† Final concentrations in the defined medium are given in weight and molar units, except for lecithin and sphingomyelin, whose fatty acid side chains are heterogeneous.

‡ Epidermal growth factor (EGF) is prepared in our laboratory as described in the text.

Trypsinization. Trypsinization is done by the previously described low-temperature method (1, 15), with the added modification that the partially loosened cellular monolayer is washed with 10 ml of solution A to remove excess trypsin while the cells are still attached to the culture surface.

Clonal Growth Assay. Clonal growth experiments are done in 60-mm-diameter plastic tissue culture dishes (Lux 5220) coated with polylysine as described (1, 16). Supplements A, B, and C are added separately to 5.0 ml of medium in each dish, which is then equilibrated for 1 hr at 37°C in the 2% CO_2 incubator before inoculation.

Cells are depleted of serum before use in a serum-free assay. Frozen cells are thawed, placed in MCDB 110 with FBSP at 5 mg/ml for 24 hr, and then into MCDB 110 with FBSP at 1 mg/ml. Prior to confluency, they are trypsinized by the low-temperature method and split 1:2 into MCDB 110 plus FBSP at 1 mg/ml. After 1 day, the medium is replaced with MCDB 110 plus FBSP at 50 μg/ml for 12–24 hr and then with unsupplemented MCDB 110 for another 12–24 hr. The serum-depleted cells are then trypsinized by the low-temperature method, counted, serially diluted in MCDB 110, and inoculated into the preequilibrated medium at a density of 200–1000 cells per dish.

The assays are incubated for 12 days at 37°C in an atmosphere of 2% CO_2 in air saturated with water vapor. The cells are fixed with 2% (wt/vol) glutaraldehyde in 0.05 M sodium cacodylate (pH 7.0) and stained with 1% crystal violet. Total colony area

per dish, which reflects both plating efficiency and colony size (growth rate), is measured with an Artek model 880 colony counter. A FBSP response curve is included as a control in each experimental series.

Serial Subculture. Serial subculture experiments are done in 25-cm^2 plastic tissue culture flasks, generally without polylysine coating, which seems to have little effect at the cell densities involved. A counted inoculum of 2.5×10^4 cells in 5 ml of medium is allowed to multiply until growth slows (final densities are somewhat lower in the serum-free medium). The cells are then trypsinized by the low-temperature method and counted. Daughter flasks are inoculated with 2.5×10^4 cells each. Number of doublings per passage is calculated from size of inoculum and cellular yield.

RESULTS

The replacement of serum with supplements A_0, B, and C_0 for clonal growth of Flow 2000 cells is illustrated in Figs. 1 and 2. When added singly, only supplement C_0 has any substantial effect. With C_0 present, however, supplement B becomes highly stimulatory. Supplement A_0 is also stimulatory, but only with C_0 and B present. The three supplements together support clonal growth that is over 60% (based on total colony area per dish) of the maximum obtainable with an optimal amount of dialyzed serum (FBSP at 1 mg/ml). These results illustrate well the principle that the first limiting factor must be supplied before a response to the next most limiting factor can be observed (3).

Serial passage of Flow 2000 cells can be carried out readily in MCDB 110 plus supplements A_0, B, and C_0. In a pilot experiment, the cells went through four serial subculturings (minimum of 12 population doublings) in the time required for controls with FBSP at 1 mg/ml to undergo six subcultures (minimum of 18 population doublings). Multiplication rates appear to be somewhat slower in the serum-free medium, but no correction was made for possible differences in attachment efficiency in these preliminary experiments.

MCDB 110 plus supplements A_0, B, and C_0 also supports clonal growth of human fetal lung fibroblasts MRC-5, IMR-90, and WI-38, and of human foreskin fibroblasts (Table 3). Results were similar with cellular inocula of 200 or 1000 cells per 60-mm Petri dish. Substantial variation in the growth response was observed from one cell strain to another. However, the cells were not all at the same population doubling number when tested, and this may have influenced the results.

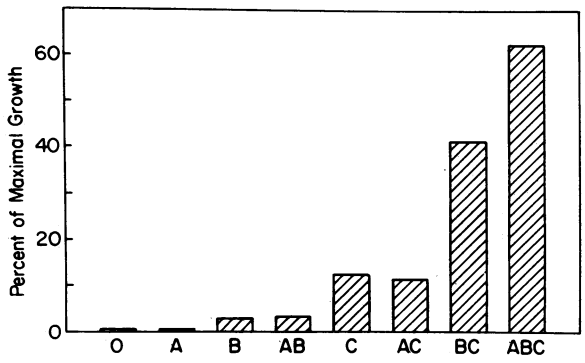

FIG. 2. Clonal growth response of Flow 2000 cells to supplements A_0, B, and C_0, singly and in combination. Clonal growth experiments were performed in MCDB 110 with an inoculum of 1000 cells per 60-mm dish and an incubation period of 12 days. The bars represent colony area per 60-mm dish as a percentage of values obtained with FBSP at 1000 μg/ml in the same experiment. The basal medium is MCDB 110. A, B, and C indicate addition of supplements A_0, B, and C_0 at the final concentrations described in *Materials and Methods*.

The two prostaglandins in supplement A_0 are responsible for most of its effect (Fig. 1*D* vs. Fig. 1*C*). Glutathione, dithiothreitol, and phospho*enol*pyruvate consistently appear to be marginally beneficial (data not shown). However, deletion of the two cyclic nucleotides has no apparent effect. They have therefore been omitted from the final version of supplement A.

Purified phosphatidylcholine alone will not replace the commercial grade lecithin in supplement B. Addition of phosphatidylethanolamine improves growth somewhat. The mixture of four purified phospholipids in supplement P-4 supports quite good growth, with a total colony area about 75% of that with supplement B and nearly 50% of that with optimal FBSP (Table 4). However, the purified phospholipids are far less stable than the commercial grade lecithin, which, despite its poorly defined lipid composition, is quite pure with regard to nonlipoidal substances. Thus, when utmost chemical purity is not critical, sup-

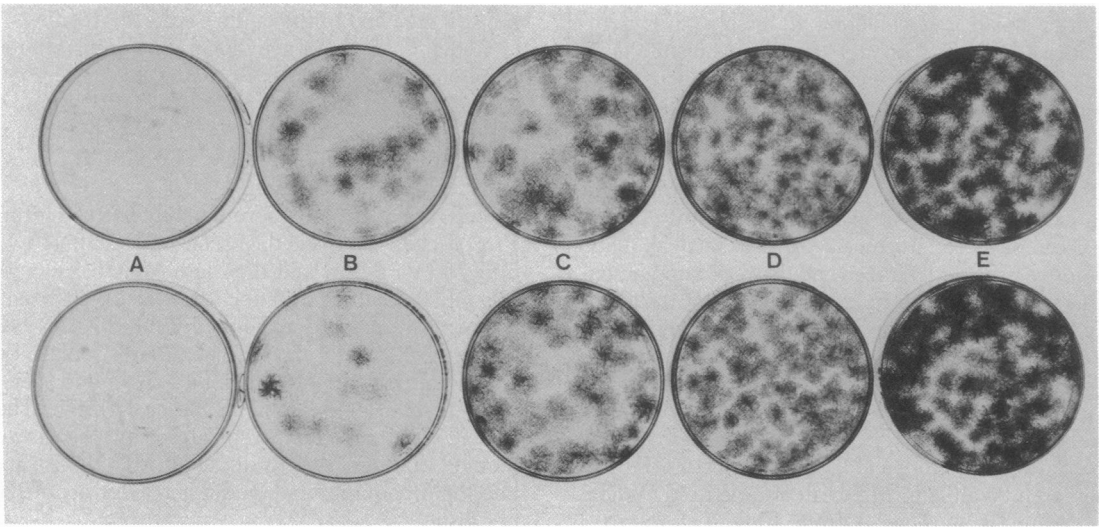

FIG. 1. Clonal growth response of Flow 2000 cells to defined supplements and to dialyzed serum. An inoculum of 1000 cells was grown for 12 days in MCDB 110 plus the indicated additives, then fixed, stained, and photographed. Duplicate plates are shown. (*A*) Control, MCDB 110 without additives. (*B*) Like *A* plus supplement C_0. (*C*) Like *A* plus supplements C_0 and B. (*D*) Like A plus supplements C_0, B, and A_0. (*E*) Like *A* plus FBSP at 1000 μg/ml (equivalent in protein concentration to 2% whole fetal bovine serum.

Table 3. Clonal growth of various lines of HDF in MCDB 110 plus supplements A_0, B, and C_0

Cell type	Passage no.	Growth*
Human fetal lung fibroblasts		
Flow 2000	15	66.3 ± 12.1
WI-38	23	42.1 ± 7.6
MRC-5	23	20.4 ± 9.5
IMR-90	20	18.0 ± 3.0
Human neonatal foreskin fibroblasts	9	90.5 ± 36.8

* The data presented are total colony area per dish for cells grown in the serum-free medium, expressed as percentage of growth of the same cell type in MCDB 110 plus optimal FBSP. The values reported are mean ± SEM for two to four assays with four replicates per assay.

plement B is preferable to supplement P-4.

Deletion of cholesterol or sphingomyelin from supplement B reduces clonal growth substantially (Table 4). Deletion of vitamin E and vitamin E acetate increases variability from one experiment to another and usually results in a slight decrease in clonal growth (data not shown). Titration of complete supplement B in the serum-free medium indicates that a total lipid concentration of 10 μg/ml is optimal (Table 4).

Titration of the components of supplement C_0 has confirmed earlier observations made in less complete media (8, 10) of major growth responses to EGF and insulin and a minor response to dexamethasone (data not shown). The optimum ranges are broad enough to allow the total protein content of the serum-free medium to be reduced below 1.0 μg/ml without affecting the overall growth response.

Replacement of serum by supplements A, B, and C requires an optimized basal medium. Essentially no growth is obtained when commercially prepared Dulbecco's modified Eagle's medium (DME), F12, or a 50:50 mixture of DME and F12 is used in place of MCDB 110. Medium MCDB 104 (1), freshly prepared in our laboratory and used with 5% CO_2 incubation, supports clonal growth of Flow 2000 cells with supplements A, B, and C. Commercially prepared dry powder MCDB 104 (custom order from GIBCO, kindly supplied to us by Paul Phillips) also works well, but only when supplemented with 5 μM freshly dissolved ferrous sulfate in addition to supplements A, B, and C. Similar results were also obtained in earlier studies involving only supplement C_0 (10).

Table 4. Response of Flow 2000 cells to lipid supplements

Lipid supplement*	Colony area †
None (MCDB 110 plus supplements A_0 and C_0)	12
P-1	15
P-2	30
P-4	48
B	63
B minus sphingomyelin	53
B minus cholesterol	38
B minus sphingomyelin and cholesterol	26
B with total lipid at 1.0 μg/ml	30
B with total lipid at 3.3 μg/ml	48
B with total lipid at 10 μg/ml	63
B with total lipid at 33 μg/ml	41

* Experimental conditions are as described in legend to Fig. 2. All components of the lipid supplements are at concentrations indicated in *Materials and Methods* except where indicated otherwise in table.
† Total colony area per Petri dish expressed as percentage of that obtained with optimal FBSP.

DISCUSSION

As used here, "defined" refers only to absence of deliberately added undefined substances. Possible requirements of HDF for contaminants in the defined medium can now be studied, but such studies are far beyond the scope of the current work (17). The history of defined media for established lines has been reviewed elsewhere (18, 19) and will not be discussed here. Four distinct phases in the development of the defined medium for HDF are reflected symbolically in the four major parts of that medium (MCDB 110 and the three supplements).

MCDB 110 symbolizes optimization of the culture medium and methodology for clonal growth of HDF with minimal amounts of FBSP. This approach, which is similar in principle to pioneering studies on permanent cell lines in the laboratories of Eagle (20), Puck (21), and others, has been reviewed in detail elsewhere (2, 3, 17, 22, 23).

Supplement C symbolizes replacement of serum with hormones, growth factors, carrier proteins, and additional nutrients. This approach, pioneered by Sato and coworkers, works well for permanent lines (5–7) and some normal cells (24, 25) but is not effective alone for HDF. However, when combined with optimized medium and techniques, it yields significant growth of HDF (8–11) and provides a culture system that is responsive to supplements A and B (Figs. 1 and 2).

Supplement B symbolizes cellular lipid requirements, which are frequently neglected in the design of defined media. Cultured cells preferentially utilize lipids from serum lipoproteins when they are available (26, 27). About one-fifth of the dry mass of HDF is composed of lipids (28). The ability of FBSP to support multiplication of HDF is inactivated by delipidation and partially restored by liposomes prepared from soybean lecithin (29).

Supplement B supplies a lipid mixture similar in composition to cellular plasma membrane lipids. Cholesterol and sphingomyelin both contribute to its multiplication-promoting activity (Table 4). Many responses of cultured cells to cholesterol have been reported (18, 30), including a response of HDF to added cholesterol in a lipoprotein-depleted medium (31). We are unaware of previous reports of responses to sphingomyelin. Liposomes are widely used in cell culture, but more often as vehicles for introducing trapped substances into cells (32) than as direct sources of nutrients (27, 28). Yamane *et al.* (33) use bovine serum albumin as a lipid carrier in a "serum-free" medium that supports excellent long-term growth of HDF but is less defined than our medium, due to its high concentration of albumin (5 mg/ml).

Possible effects of vitamin E on cultured cells remain controversial (34). Free vitamin E is added to supplement B as an antioxidant, and vitamin E acetate is included as a stable form that is inert until it is hydrolyzed after entering the cell.

Supplement A symbolizes the beginning of a long process of "fine tuning" of the defined medium culture system for HDF. Prostaglandins E_1 and $F_{2\alpha}$, which are its most active components, have previously been found beneficial in a number of culture systems (7, 22), including our own preliminary studies with small amounts of FBSP (35). The other three components of supplement A all appear to be marginally beneficial. Because our laboratory is at an altitude of 1650 meters, with reduced oxygen tension relative to sea level, the slight effects of reducing agents that we observe here may become more significant at lower altitudes.

We anticipate that additional growth requirements for HDF in defined media will be identified in the future. For example, Phillips and Cristofalo (9, 11) have reported that platelet-derived growth factor is needed for monolayer density equivalent

to that obtained with serum. Preliminary studies in our laboratory have shown that purified platelet-derived growth factor (kindly supplied by Bengt Westermark) increases cellular density within individual colonies of HDF when added to the defined medium on day 7 of a 12-day clonal growth assay.

Many other studies remain to be done on growth of HDF in the defined medium, including further quantitative optimization, an evaluation of the possible role of contaminants, and precise identification of the multiplication-promoting substances in serum that have been replaced by specific components of the defined medium, However, the need for such studies does not lessen the significance of reliable and reproducible growth of normal human cells in a medium that contains no deliberately added undefined substances. The principles that led to development of the defined medium for HDF can now be applied with confidence to other cell types. Approaches similar to those described here have already resulted in clonal growth in defined media of human epidermal keratinocytes (36) and rabbit ear chondrocytes (37).

The results presented in this paper are the culmination of research that for one of us (R.G.H.) has extended over more than 20 years. Special acknowledgements are due to Dr. Theodore T. Puck, who first encouraged and supported this effort, and to Dr. Wallace McKeehan, Kerstin McKeehan, Linda Miller, Susan Hammond, Cathy Malhotra, Kathleen Bowen, Steven Mease, and Dennis Genereux for their roles in providing the foundations for the currently reported experiments. We thank Debra Lancaster for excellent technical assistance and Karen Brown for preparing the manuscript. This research was supported by National Institutes of Health Grants CA 15305 and GM 26455.

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