CLONAL GROWTH IN VITRO OF HUMAN CELLS WITH FIBROBLASTIC MORPHOLOGY*, ‡

Comparison of Growth and Genetic Characteristics of Single Epithelioid and Fibroblast-Like Cells from a Variety of Human Organs

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Plates 9 to 12

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In previous papers (1–3) we have described methods for plating single human cells so that each multiplies in isolation to form a well defined colony which constitutes a clonal population. The procedure is simple and quantitative, and completely comparable to the standard plating procedure used by microbiologists for growth of colonies from single bacterial cells. In the work published thus far we have described application of this method only to cells of epithelioid morphology. The present communication demonstrates that fibroblastic cells, although requiring more stringent care, can also be plated by this technique. Experiments comparing growth and genetic properties of these two cell types obtained from a variety of human organs are presented and some implications of these results for the problem of differentiation are discussed.

Definitions.—The terms, *clone, plating efficiency,* and *parental strain* are used as previously defined (2, 4, 5). *Fibroblastic morphology:* Elongated form of a glass-attached cell, which is spindle-shaped or possesses multiple, needle-like extensions of the protoplasm, as shown in Fig. 1 *a*. Colonies of such cells display rough edges. *Epithelioid morphology:* Cell form consisting of polygonal bodies whose angles are roughly equal in size, as shown in Fig. 1 *b*. These cells usually pack together fairly tightly, display less surface area and are less migratory than fibroblastic cells (2). In this paper these two terms refer only to the morphologic appearance of the glass-attached cells, without implications concerning their origin *in vivo*. *Genetic* or *hereditary* character: A character, the potentiality for which is transmitted to all the asexual progeny of the original individual even after many passages in an environment which does not permit development or expression of the character. Not all genetic potentialities are *genic*; i.e. under exclusive control of specific nuclear genes. (See discussion.)

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TABLE I

Composition of Solutions Employed in This Study

	gm./liter		gm./liter
A1. Saline A		*A2.* Hanks's saline	
Phenol red	0.02	Phenol red	0.02
NaCl	8.00	NaCl	8.00
KCl	0.40	KCl	0.40
Glucose	1.00	$CaCl_2 \cdot 2H_2O$	0.18
$NaHCO_3$	0.35	$MgSO_4 \cdot 7H_2O$	0.20
		$Na_2HPO_4 \cdot 7H_2O$	0.09
		KH_2PO_4	0.06
		Glucose	1.00
		$NaHCO_3$	0.35

B1. Trypsin solution, 0.03 per cent (0.3 gm. trypsin (1-300) to 1 liter of saline A)

B2. " " 0.05 " " (0.5 " " " " " " " " ")

Trypsin (1–300) was obtained from Nutritional Biochemicals, Cleveland.

Synthetic Mixtures for Incorporation in Growth Media

	gm./liter		gm./liter
C1.		*C1.* (*continued*)	
L-Arginine	0.0375	Inositol	0.0010
L-Aspartic acid	0.0300	Biotin	0.0001
L-Cystine	0.0075	Calcium pantothenate	0.0030
L-Glutamic acid	0.0750	Choline	0.0030
Glycine	0.100	Folic acid	0.0001
L-Histidine	0.0375	Niacinamide	0.0030
DL-Isoleucine	0.0250	Pyridoxine	0.0005
L-Leucine	0.0250	Riboflavin	0.0005
L-Lysine	0.0800	Thiamin	0.0050
L-Methionine	0.0250	Glutamine	0.2000
β-Phenyl-L-alanine	0.0250	Hypoxanthine	0.0250
L-Proline	0.0250	Glucose	1.2000
L-Threonine	0.0375	Phenol red	0.0050
L-Tryptophan	0.0200	NaCl	7.00
L-Tyrosine	0.0400	KCl	0.20
DL-Valine	0.0500	$CaCl_2 \cdot 2H_2O$	0.14
		$MgCl_2 \cdot 6H_2O$	0.10
		Na_2HPO_4	0.23
		KH_2PO_4	0.10
		$NaHCO_3$	2.24
		Penicillin	0.250
		Streptomycin	0.250

Biological Fluids Used for Incorporation in Growth Media

D1. Human serum—obtained from George Washington Carver Foundation, and pretested as explained in text

D2. Horse serum—obtained from Colorado Serum Company

TABLE I—*Concluded*

D3. Chick embryo extract—50 per cent. The embryo extract employed in this study was prepared by the procedure of Bryant, Earle, and Peppers (9) but with the following modifications: (*a*) 10 day old chick embryos were used; (*b*) The final concentration of hyaluronidase was reduced to 150 T.R. units per 100 ml. of extract; (*c*) The treated extract was ultracentrifuged 3 hours at 36,000 *g*; and (*d*) The ultracentrifuged supernatant was filtered through a Selas filter candle: No. 02 porosity.

Composition of Growth Media Employed

E1. "Fibroblastic" growth media—containing embryo extract. (That under *E1 a* was used most frequently; that described under *E1 b* was soon superseded.)
E1 a. Synthetic mixture *C1*, 10 per cent
Human serum (*D1*), 17 per cent
Embryo extract (*D3*), 4 per cent
Hanks's (*A2*), 69 per cent
E1 b. Changs's medium (4):
Human serum (*D1*), 20 per cent
Embryo extract (*D3*), 5 per cent
Hanks's saline (*A2*), 75 per cent
E2. "Epithelioid" growth media: contain no embryo extract
E2 a. Synthetic mixture (*C1*), 10 per cent
Human serum (*D1*), 20 per cent
Horse serum (*D2*), 10 per cent
Hanks's (*A2*), 60 per cent
E2 b. Same as *E2 a*, but without horse serum, and with 10 per cent or 20 per cent human serum.

Materials

All the solutions used in this work are listed in Table I. The solutions there designated "fibroblastic" and "epithelioid" media, and which differ mainly in the presence of embryo extract, are formulations which have been found fairly consistently to promote the best growth of the respective cell types, as single cells. As will be shown later, however, all of the epithelioid cells can grow in the "fibroblastic media," though usually at reduced rates, and some fibroblastic cells can form colonies in "epithelioid" media, in this case, however, with a much lower growth rate. All incubations were performed at 38°C. in a humidified atmosphere of 5 per cent CO_2 as previously described (3). All of the fibroblastic cell strains employed were isolated in this laboratory directly from human tissues, except for the MAF strain of human embryonic origin supplied by Microbiological Associates, Inc., Bethesda, Maryland.

Details of the Techniques Employed

1. Preparation of Dispersed Cell Suspensions from Human Biopsy Specimens and Their Transfer to Growth in Vitro.—Tissue samples were obtained from a variety of human organs, either in the course of routine surgical procedures or from biopsy performed on normal volunteers. The cells here considered came only from normal subjects or from patients with no evidence of any neoplastic process. The tissue was removed aseptically and transferred to 10 cc. of any of the fibroblastic growth media (Table I, *A*, *B*, or *C*). The amount of material used never exceeded 1 gm. wet weight, and frequently was less than 200 mg. Physical trauma to the tissue was always minimal.

Thin specimens, as in the case of skin or amniotic membrane, were not subdivided further.

Thicker ones were minced sterilely in a Petri dish with maximally sharp scissors into slices 1 mm. or less in thickness.

The bathing solution was removed with a pipette, and the sample washed twice with saline A (Table I, *A1*). The tissue was then covered with 0.05 per cent trypsin (Table I, *B2*) and placed in a 38°C. bath for 15 to 45 minutes. Trypsinization was judged to be sufficient when the solution had become faintly cloudy, and the edges of the tissue had acquired a transparent, gelatinous appearance. 1 cc. quantities of this essentially monodisperse suspension were then added to each of four culture bottles, each containing 9 cc. of the appropriate growth medium, which in most of the experiments here described corresponded to composition *E1 a* of Table I. The bottles were closed with sterile cotton plugs and incubated at 38°C. in 5 per cent CO_2.

Within 6 hours most of the free cells had become attached to the glass surface. Incubation was interrupted only for daily microscopic examination of the cells, and for 48 hourly replacement of the medium. The cells at first remain rounded and may be dormant for varying periods. Sooner or later, however, growth is initiated. The time taken for growth to start varied with the different tissue samples. In some cases, *foci* of active cell multiplication were visible within 48 hours. In at least one case the cells remained rounded and inert for 3 weeks, after which almost every cell, within 48 hours, became stretched and entered into active reproduction. Unquestionably profound adaptive processes go on during this period in which the cell makes the transition from a possibly non-multiplying condition in the body to a state of very high reproductive rate *in vitro*. While the factors controlling this lag period are not yet defined, it is our impression that this dormant interval is smallest when the cell number in each bottle is large and when the tissue has not been traumatized in the course of its removal from the body. By planting the dispersed cells in four different bottles, the probability of loss of the strain through microbial contamination is made negligible. In a series of 12 successive tissue cultivations from a variety of non-malignant human organs carried out in this laboratory, not one failed to initiate healthy growth of fibroblastic cells. Growth of epitheloid cells from such tissue biopsies is discussed later in this paper.

2. Maintenance of Cultures in a Healthy, Actively Multiplying State.—It is a common experience in tissue culture that freshly isolated human cells can be readily induced to multiply temporarily. After a period of weeks, the growth of such cultures usually declines and eventually stops. Because of this pattern of events it has been proposed that no cell shall be considered as established in tissue culture until it shall have been maintained for at least 6 months in an actively growing state (6).

Similar behavior of such cultures was observed in our early experiments. After approximately 1 or 2 months of cultivation by the routine procedure employed for epitheloid cells, (involving untested media, and no more than one or two medium changes per week) cells were observed to decrease their rate of multiplication, and become enlarged, acquiring rough edges and granular, cytoplasmic bodies. If this regime continues, the cells become swollen and distorted, as shown in Fig. 2 *a*, and eventually die.

This sequence of events strongly suggests an inadequacy of the nutrient medium due to presence of toxic substances or to deficiencies in the required concentrations of nutrilites. Hence, the following general procedure was devised, aimed at correcting for either or both of these contigencies: (*a*) Toxic media were identified for rejection by subjecting aliquots of each batch of embryo extract and human serum to test before use by observing whether, when incorporated in fibroblastic growth medium (Table I), they would promote growth of colonies from single fibroblast cells, by the plating procedure described in subsequent paragraphs. (*b*) The medium was changed regularly in all bottles every 48 hours. Thus, nutrilites present in suboptimal amounts in the medium are continuously replenished with each change. (*c*) The cells were not allowed to reach too high a density in each bottle, but were trypsinized and

subdivided by or before the time when they cover 30 per cent of the available surface. As a routine measure, each bottle was trypsinized at least once a week. (*d*) In inoculation of new bottles, at least 10^4 cells were always introduced in order to insure the additional mutual support which cells can provide each other under these conditions (7, 8, 1).

Of all these measures, possibly the most important is the screening of the batches of embryo extract. While control experiments have shown appreciable differences in the ability to support cell growth of sera prepared from different human donors, a far greater range of variability is exhibited by successive batches of chick embryo extract, presumably prepared by exactly the same procedure from similar batches of embryonated eggs.

When these measures were faithfully observed, fibroblastic cell lines were readily maintained in active multiplication for long periods, and have been carried in this laboratory for as much as a year after their initial isolation. Experience has shown that even small deviations from the 4 step routine listed in the preceding paragraph, may endanger the culture. The first sign of such degeneration is the enlargement of the surface area exhibited by the cells. Eventually other signs of the non-multiplying state assert themselves. When such symptoms become manifest, the only recourse known to us is to return to the prescribed routine. Bottles may have to be trypsinized and their contents combined rather than subdivided, in order to achieve the proper inoculum of 10^4 cells per bottle. A further difficulty at this point is the fact that cells which have become abnormally stretched and distorted, display a high mortality upon trypsinization, doubtless because of the greater opportunity for strain in the release of such an extended surface from its bond to the glass. Weeks or even months of constant nursing may be required to effect the return of some cultures to the rapidly multiplying state. However, we have succeeded several times in accomplishing the reversal of this degenerative process. An example of such a revival is shown in Figs. 2 *a* and 2 *b*. Such experience seems to warrant the conclusion that current nutrient media are at best only marginally adequate for maintenance of many human fibroblastic cells in the actively multiplying state. Nevertheless, the method we have outlined has been successful in the continued cultivation of fibroblastic cells from more than 12 freshly biopsied samples which were taken from human skin, muscle, spleen, amnion, bone marrow, heart, liver, lung and brain. Almost never have we lost a culture which has been conscientiously cultivated by the method described. This procedure is admittedly exceedingly laborious, but it appears to furnish a reliable method of fibroblastic cell cultivation. Studies are in progress to achieve better definition of the nutritional needs of these cells in the hopes that the labor required may be diminished.

3. Single Cell Plating.—Monodisperse suspensions are obtained from the farmed cells by the following procedure: Growth medium is pipetted off from the bottle to be harvested, and the cells washed once at room temperature with 5.0 cc. of saline A, which is then removed and replaced with trypsin solution (*B1* of Table I). After 10 minutes of incubation at 38°C. with occasional agitation, the tryptic action is ended by dilution with an equal volume of growth medium at room temperature. An aliquot of this suspension is removed for determination of cell density in a hemocytometer. Then appropriate dilutions are made in growth medium and an aliquot containing the desired number of single cells is plated in a 60 mm. Petri dish containing 5.0 cc. of fibroblastic growth medium, and is incubated.

When the plating procedure is to involve the use of feeder cells (2), these are first prepared by exposure of a bottle containing a confluent monolayer of cells to a dose of 2500 to 4000 roentgens of 200 to 240 k.v. x-rays. The monolayer is then trypsinized and 10^5 cells added to each plate along with the aliquot of the live cells whose growth potential is to be titrated. Alternatively, the cells for the feeder layer can be added to each plate before irradiation, which is then accomplished *in situ*, after which the inoculum of living cells is introduced. It is obvious that whenever a feeder cell layer is employed, careful controls consisting of plates with feeder cells alone must be included in the experiment in order to eliminate the possibility of colony formation arising from surviving cells in the feeder layer.

Usually 10 to 12 days of incubation is advisable for optimal colony development of fibroblasts. Whenever a "feeder" cell layer is employed, it is necessary to change the medium on the 6th day. On completion of incubation, the growth medium is poured off, the plates rinsed twice with physiological saline, and the colonies fixed by exposure to 10 per cent neutralized formalin for about 5 minutes at room temperature. The cells may then be stained with standard giemsa or hematoxylin-eosin solutions.

EXPERIMENTAL RESULTS

1. Growth of Single Fibroblastic Cells into Colonies by the Standard Plating Procedure.—Typical results of plating single fibroblastic cells from a variety of human organs, by means of the procedures described, are shown in Fig. 3. The developing colonies are readily recognizable despite their characteristic rough outlines, resulting from the elongated shape and migratory tendency of these cells. The colonies often contain swirls and configurations resembling locks of hair, occasioned by a tendency of the elongated cells to align in parallel. The plating efficiencies obtained with fibroblastic cells are determined primarily by the state of the culture at the time of harvest, and in this way reflect the much greater sensitivity of these as compared with epithelioid cells, to degeneration unless carefully tended. Thus, cells which are in the optimal growth state usually produce colonies with fairly high efficiency (about 60 per cent) when plated directly into the nutrient medium. Cells which are in somewhat less healthy condition yield very low plating efficiencies which, however, can often be raised considerably by furnishing an x-irradiated "feeder" layer to support the single cell inoculum (See Fig. 3 *e* to Fig. 3 *h*). Cultures allowed to degenerate like those of Fig. 2 *a* give no colonies when single cells are plated.

The distinctness of these colonies is more readily lost through coalescence than is the case with the more compact colonies of epithelioid cells, a tendency which also contributes to reduction of the apparent plating efficiency of fibroblastic cells, whenever more than 50 are plated on the same Petri dish.

Fig. 4 presents a typical plating of single cells from an epithelioid strain for comparison. A summary of some of the plating efficiencies so far achieved with various fibroblastic cell strains is presented in Table II. The data reveal that good plating efficiencies (40 to 60 per cent) are obtained from single human fibroblastic cells taken from a variety of non-cancerous organs, from cells originating either in adult or children's tissues, and from cells previously cultivated for long or short periods *in vitro*.

In general, the growth rate of these single cells approaches that previously observed for the epithelioid cells; *i.e.*, a generation time in the neighborhood of 20 to 24 hours (2).

2. Preparation of Clonal Stocks and Stability of the Fibroblastic Trait.—Well isolated colonies can be picked and made the basis for establishment of clonal

stocks by means of the procedure previously described (2). Again, the establishment of fibroblastic cell clones requires more care than do epithelioid cells. Fibroblastic colonies should be permitted to attain a size of several thousand cells before picking, for optimal reliability. In other respects, the results of such colony picking are the same as those reported with epithelioid cells (2).

Of particular importance is the fact that the cells of such clonal strains can retain their characteristic fibroblastic morphology indefinitely. For example, clonal cells from a normal human skin (Hansen) have retained their

TABLE II

Representative plating efficiencies of single cells from a variety of human, non-malignant organs. Some of these cells, like the MAF fibroblast, had been previously maintained in tissue culture for many months. Others, like the spleen and skin cells, were plated successfully as soon as enough cells had reproduced to give accurate counts in a hemocytometer, a matter of 2 to 5 weeks after the initial biopsy.

		Plating efficiency		
Organ of origin	Age of subject from whom biopsy was taken	Epithelioid medium	Fibroblastic medium	Epithelioid medium + feeder layer of the same cell irradiated with 2,000 r
	yrs.	*per cent*	*per cent*	*per cent*
	I. Fibroblastic cells			
Amnion (Hoag)	Over 20		65	
Skin, muscle (MAF)	Embryonic	0		25
Skin, (Hansen)	Over 20	0–2	46	
Spleen, (Mendoza)	11		56	
	II. Representative Epithelioid Cell for Comparison (2)			
Conjunctiva		82	81	

identifying morphology after more than 50 generations of growth. When such clonal cells are again plated, each one again forms a completely characteristic fibroblastic colony. It may be concluded that the morphologic character which differentiates these clonal cells from those of epithelioid morphology previously described (2, 3) is a stable genetic trait under the conditions of these experiments.

3. Comparison of the Behavior of Human Cells with Epithelioid and Fibroblastic Morphologies.—With the demonstration that stable clonal cell lines of both morphological types can be established and that single cells of each behave like microorganisms in their ability to reproduce indefinitely in isolation, a study of the differences in behavior of these two different lines of human cells can be undertaken.

(*a*) *Cellular and colonial morphology:* The clonal fibroblastic cell lines have never reversibly altered their morphology, but always exhibited only an elongated, spindle-shaped cell. In contrast, clonal epithelioid cell lines can vary their shape considerably, depending on the amount of human serum of certain kinds present in the medium, as we have previously shown (2). In any medium available to us the difference between fibroblastic and epithelioid cells, as these terms are here employed, is unequivocal. However, since epithelioid cells increase their elongation in the presence of large amounts of human serum or in the presence of embryo extract, clearest morphological differentiation of these cell types is obtained in media of the *E* series of Table I. Regardless of their previous media of cultivation, whenever single cells of each type are plated in parallel in the same medium, every one of the developing colonies assumes a morphology unequivocally characteristic of its own type, as shown by comparison of Figs. 3 and 4. We conclude that the cellular morphological characteristics which differentiate epithelioid from fibroblastic cells are controlled by 2 factors: a genetic component which determines whether a cell is fibroblastic or epithelioid; and a molecular environmental factor which fixes the degree to which the more versatile epithelioid cells will stretch on glass.

Trypsinized fibroblastic cells are completely spherical and resemble in volume the epithelioid cells we have studied. Thus, a clonal strain of a typical fibroblastic skin cell (Hansen) yielded on trypsinization, spheres with a mean diameter of $21.0 \pm 3\mu$, which is equivalent to a volume of $4.7 \times 10^3\mu^3$, a value higher but still comparable to that of about $2.3 \times 10^3\mu^3$ obtained for a variety of epithelial cells (2).

(*b*) *Nutritional Requirements for Growth:* The nutritional requirements of fibroblastic and epithelioid cells reveal interesting differences. All the epithelioid strains studied by us (originating from normal human conjunctiva, appendix, kidney, liver, amnion, and lung, and from a cervical carcinoma) can be plated regularly and successfully as single cells in nutrient media which contain no embryo extract (Solutions *E2 a* or *E2 b* of Table I). Most of the fibroblastic strains here studied give low efficiencies of single cell plating in this medium, unless it is supplemented as with a feeder layer or embryo extract, as shown in Fig. 3. When an occasional fibroblastic cell produces colonies in media lacking embryo extract, its growth rate is reduced over that which is obtained in the enriched medium.

It is of interest in this connection that some fibroblasts like MAF will grow indefinitely with apparently maximal rate in this limited medium provided that massive inocula are employed. This behavior explains why a feeder layer of the same cell type can also support growth of single cells in an otherwise deficient medium. The significance of such differences in growth requirements between single cells and mass cultures has been discussed elsewhere (8).

The epithelioid cells we have so far studied can grow in either the fibroblastic or epithelioid medium shown in Table I, but their growth usually is less rapid in the medium containing the embryo extract.

(*c*) *Sensitivity to Action of Trypsin:* The highly stretched fibroblastic cells, with their greater area of contact with glass, might be expected to be more refractory to dispersal by trypsin than the columnar epithelioid cells which make contact only at their relatively narrow base. This supposition was confirmed by experiments in which cells of each type were treated with different concentrations of trypsin at 38°C., and then observed for evidence of liberation from their bond to the glass surface. Thus, epithelioid cells from human conjunctiva were observed to round up into spherical shapes at trypsin concentrations $\frac{1}{5}$ to $\frac{1}{10}$ that required to produce the same degree of change in an equivalent time with fibroblastic cells from human skin.

(*d*) *Ability to withstand Adverse Conditions:* The enormously greater ruggedness of epithelioid, as opposed to fibroblastic, cell types to suboptimal growth conditions, which has already been noted, constitutes a real difference in kind. The former cells require much less care in their cultivation, and regularly yield plating efficiencies in the neighborhood of 100 per cent, even from stock bottles which have gone without medium changes for periods of 2 weeks or more, an interesting contrast to the delicate fibroblastic cells.

4. Origin of Fibroblastic and Epithelioid Cells—Demonstration of Their Joint Presence in Tissues Taken from the Same Individual.—The processes leading to the formation of clonal cell lines which display stable hereditary differences when grown *in vitro* are of fundamental importance. On the basis of our findings that single cells of epithelioid strains will grow in the simple media of Table I, *E2*, while those of fibroblastic type require supplementation as by embryo extract, an experiment was performed seeking to demonstrate the presence of cells of both hereditary types in tissue specimens taken from a single individual.

Monodisperse cell suspensions were prepared in the standard manner from approximately 100 mg. of human lung, skin, and muscle obtained from an 11 week old, presumably normal fetus resulting from surgical, therapeutic abortion. Equal volumes of the dispersed cells were placed in bottles containing fibroblastic and epithelioid growth media, respectively, and incubation with standard medium changes was initiated. All the cell lines produced luxuriant growth of fibroblastic cells in the "fibroblast" medium, but only the lung cells grew appreciably in the "epithelioid" medium. The behavior of the lung cells in these two media was noteworthy. Within a week, a marked difference in the cell composition of the two types of bottles was obvious: The bottle with "fibroblast" medium displayed only massive growth of fibroblastic cells. That with "epithelioid" medium contained many small patches of fibroblastic cells, but between these areas were studded highly compact aggregates

of typically epithelioid cell growth, as shown in Fig. 5. The cells taken directly from the tissue attach to the glass surface within a matter of hours, whereas their multiplication time in these media requires approximately a day or more. Since the epithelial colonies were distributed all through the bottle, it may be concluded that the potentiality for this character was present in large numbers of the tissue cells before they had reproduced *in vitro*.

Single cells from bottles containing the mixture of cell types were replated in small numbers in each medium. Separate colonies of fibroblastic and epithelioid morphologies, respectively, arose from the single cells plated, as shown in Fig. 6. Such well isolated colonies were picked and subcultured. The resulting clonal stocks completely maintained their morphological integrity, during subsequent multiplication for approximately 20 generations. The cultures from skin and pectoral muscle also served as sources of clonal stocks which displayed only fibroblastic properties.

5. Differences among Fibroblastic Cells from Different Organs.—Indications exist already that cells with fibroblastic morphology are not all identical. Some differences in nutritional behavior of massive cell populations have already been observed. Some interesting distinguishing morphological characteristics as well as certain differences in virus susceptibility, have also been observed by us. Studies comparing biochemical and other metabolic properties of clonal cell lines isolated from a variety of human organs are under way.

DISCUSSION

The experiments here described illustrate how single cell techniques can be used to determine whether or not a difference in behavior among members of a mammalian cell population constitutes evidence of a genetic difference in the specific sense in which the term has been here defined. Conclusions drawn only from experiments involving massive cell populations must always be unsatisfactory because of the opportunities for cell-cell interaction to obscure the situation. Thus, the fact that a certain characteristic behavior is exhibited by a large population of cells, whether actively multiplying or not, can never, of itself, be regarded as establishing existence of a specific genetic character, no matter how long this behavior is perpetuated in the population. Nor can changes in such a hitherto stable characteristic be accepted as evidence of a change in genetic constitution of any particular cell type. The given characteristic may be a reflection of cooperative action between parts of the population differing in genotype or in physiological state. These relationships might persist unchanged under some conditions of growth, but under others might alter so that the proportions of the cells in the different possible states would change. As a result of such a changed distribution, the gross behavior of the ensemble might undergo macroscopic alteration. In tissue culture procedures in particular, in which the media almost invariably contain natural products whose composition is not subject to control, the opportunities for inadvertent

changes in growth conditions are omnipresent, even in the most carefully regulated laboratory. Thus, the reports from many tissue culture laboratories of the incidence of spontaneous or induced changes in behavior of their cell strains must be interpreted with great caution with respect to the nature of the underlying mechanism.

The single cell plating technique makes possible definition of cellular genetic characteristics with exactly the same precision (and limitations) which has been attained in the procedures of microbiological genetics: Clonal stocks can readily be prepared from any cell in a population; single cells of such stocks can be plated, and from examination of the isolated colonies which develop, the proportion of the cells of this clonal population which possess the characteristic in question can be determined; the persistence of this distribution among the single cells of the population throughout many generations of growth under various conditions can be tested. If stable differences under these conditions in all the single cells of the two clonal strains can be demonstrated, the conclusion may be drawn that the difference is a true genetic one. The present experiments demonstrate such genetic differences between several fibroblastic and epithelioid cell lines isolated from normal human tissues.

Demonstration of the existence of a true hereditary difference in cell strains still leaves many questions to be answered. The difference may be due to change in nuclear constitution, like those which occur in normal embryonic differentiation as demonstrated by the elegant experiments of Briggs and King involving nuclear transplantation of cells in frog embryos (13). However, the effects here described could equally well be due to the action of cytoplasmic genetic determinants, or to a self-sustaining kind of interaction between nuclear and cytoplasmic apparatus in cells of a constant genotype, the possibility for which has been demonstrated in control of the ciliary antigens of *Paramecium* (10). The possibility that transducing viruses, transforming principles, or other neoclassical genetic mechanisms are involved must also be investigated. The use of single cell plating procedures makes possible application of the conceptual and experimental approaches, that have proved so illuminating in work with independent microorganisms, to the study of the nature of the cellular changes which underlie mammalian differentiation.

The experiment in which it was demonstrated that fibroblastic and epithelioid cell lines can be isolated from the same tissue specimen is also important in illuminating dynamics of the appearance of these two cell types in tissue culture. In a medium favoring fibroblast multiplication, the epithelioid cells are at a selective disadvantage. Moreover, the somewhat stronger migratory tendency of the fibroblastic cells causes them to fill up all the available space, and therefore to restrict sharply the growth of epithelioid centers. Thus, normally, tissue cells planted in "fibroblastic" medium eventually yield stocks consisting entirely of fibroblastic cells. If, however, a medium is employed which confers a degree of selective advantage on the

epithelioid cells, these can multiply and may be isolatable as clonal stocks, as was done here.

Experiments are in progress to develop more effective differential media which may permit routine separation of epithelioid and fibroblastic cell strains in many human tissues.

It would be unwise at this stage to attempt to identify cells of "fibroblastic" morphology *in vitro* with the specific fibroblasts of mesenchymal origin in the body; or to claim that "epithelioid" cells are directly descended from body epithelium. The present experiments permit only the conclusion that at least two factors govern the morphological behavior of cells grown attached to a glass surface: A stable, hereditary determinant which fixes whether the cell is or is not unvaryingly fibroblastic in the media here described; and a molecular environmental factor which determines the degree of compactness assumed by the non-fibroblastic cells.

The reliability of the procedures here described now makes feasible cultivation *in vitro* of cells taken from a variety of organs of large numbers of human subjects. The use of the single cell plating method affords more accurate methods than have been heretofore available for measurement of growth rate, nutritional requirements, and other behavior of such cell populations (11). These techniques now appear particularly well suited to screening studies attempting to characterize the cellular basis of a variety of human, metabolic diseases (12).

SUMMARY

A methodology has been described for reliable cultivation *in vitro* of dispersed fibroblastic cells obtained from normal human organs. The procedure has permitted establishment of stable cell lines from almost every sample taken, among which the following organs were represented: skin, spleen, amnion, lung, liver, bone marrow, brain, muscle, and heart. Equally good growth has been achieved with cells from embryonic or adult tissues.

The methods previously developed whereby single cells plated in Petri dishes grow into isolated macroscopic colonies can successfully be applied to the plating of human fibroblastic stocks. Plating efficiencies in the neighborhood of 50 to 60 per cent are readily achieved with such strains. The resulting colonies can be picked and clonal stocks established.

Fibroblastic morphology is maintained in the colonies arising from every single cell of such clonal stocks. All of the single cells from epithelioid clonal strains also maintain their integrity throughout repeated subculture. Since the difference between clonal stocks of these two types is always maintained whenever the respective single cells are plated in the same medium, regardless of the previous history of these stocks, it may be concluded that a true genetic difference exists in these cell lines.

In addition to the morphological differences between epithelioid and fibro-

blastic cell strains, the latter have more demanding nutritional requirements for single cell growth. Thus, single cells of fibroblastic lines almost never produce colonies with high efficiency unless the growth medium which is sufficient for epithelioid cells is supplemented with embryo extract, or a cell feeder layer. Fibroblastic cells are also more resistant to tryptic digestion of the bond uniting the cells to glass surfaces.

By use of differential media, growth of both fibroblastic and epithelioid cells, respectively, has been obtained, from dispersed single cells obtained by trypsinization of a specimen of human embryonic lung.

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EXPLANATION OFPLATES

PLATE 9

FIG. 1: Demonstration of the identifying morphological characteristics of human cells growing *in vitro* in fibroblastic and epithelioid cell configurations. 10^5 cells of each clonal stock were placed in a petri dish and incubated for 24 hours, after which the cells were fixed and stained. The same medium was employed in each case—*E2 a* of Table I.

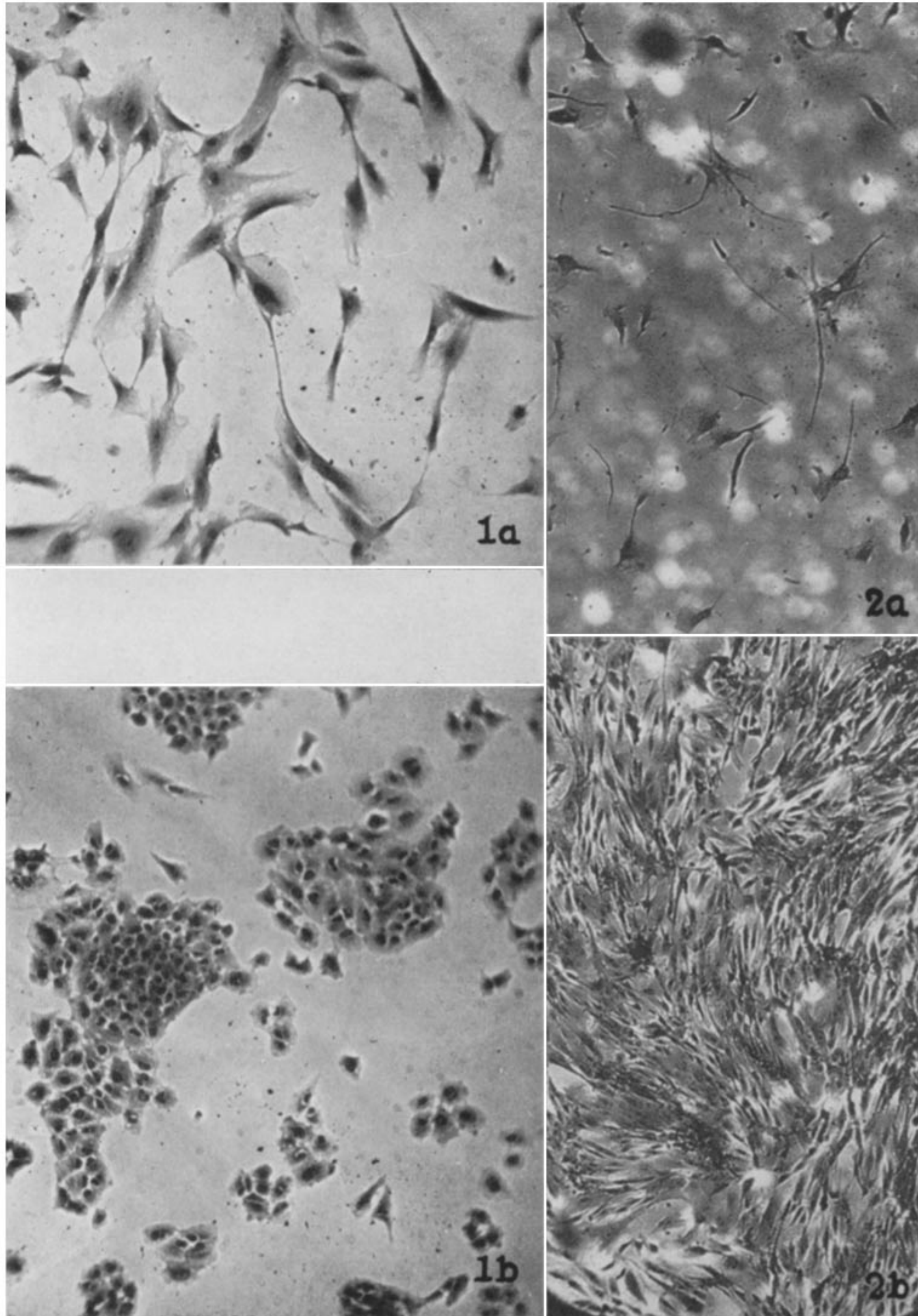
FIG. 1 *a*. Fibroblastic cells from a clonal strain originating from normal human skin. × 100.

FIG. 1 *b*. Epithelioid cells from a clonal strain originating from a human cervical carcinoma. × 100.

FIG. 2. Demonstration of the more constant care required by human fibroblastic cells as contrasted with epithelial cells of our experience, and of the fact that degenerating cultures can be restored by the 4 step procedure outlined in the text.

FIG. 2 *a*. Typical field in a bottle containing fibroblastic cells of a human spleen (Mendoza) maintained by the regimen consisting of 4 day medium changes with medium not previously tested. This procedure produces healthy and sustained growth of human epithelioid cells, but causes fibroblastic strains to degenerate as shown. × 140.

FIG. 2 *b*. Typical field in a bottle like that shown above, which was subsequently switched to the 4 step schedule described in the text. In about 3 weeks, healthy, sustained growth had been restored. × 140.



(Puck *et al.*: Clonal growth of human fibroblastic cells)

PLATE 10

FIG. 3. Typical colonies arising from plating of single human cells with fibroblastic morphologies, plated by means of the techniques described.

FIG. 3 *a*. Colonies of human spleen (Mendoza). × 1. Cells were plated in "fibroblastic medium." No feeder layer.

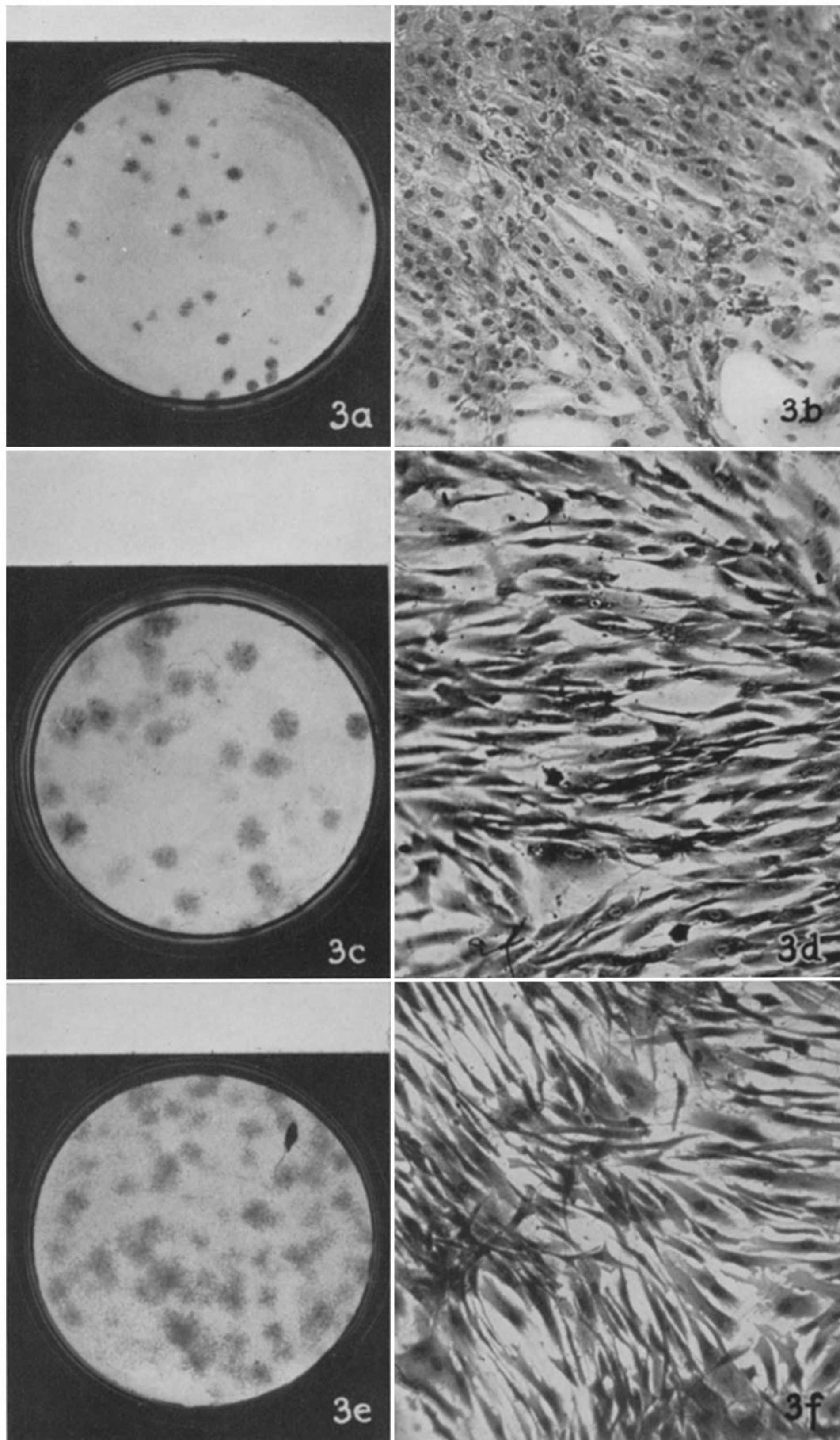
FIG. 3 *b*. Enlargement of a portion of Fig. 3 *a*. × 100.

FIG. 3 *c*. Colonies of human skin (Hansen), plated as in Fig. 3 *a*, without feeders. × 1.

FIG. 3 *d*. Enlargement of a portion of Fig. 3 *c*. × 100.

FIG. 3 *e*. Colonies of human skin (Spoor), plated in medium lacking embryo extract, but supplemented with a feeder layer of MAF fibroblasts. × 1.

FIG. 3 *f*. Enlargement of a portion of Fig. 3 *e*. × 100.



(Puck *et al.*: Clonal growth of human fibroblastic cells)

Plate 11

Fig. 3 *g*. Colonies of human fibroblast MAF, plated without embryo extract but with a feeder layer of the same cells. × 1.

Fig. 3 *h*. Enlargement of a portion of Fig. 3 *g*. × 100.

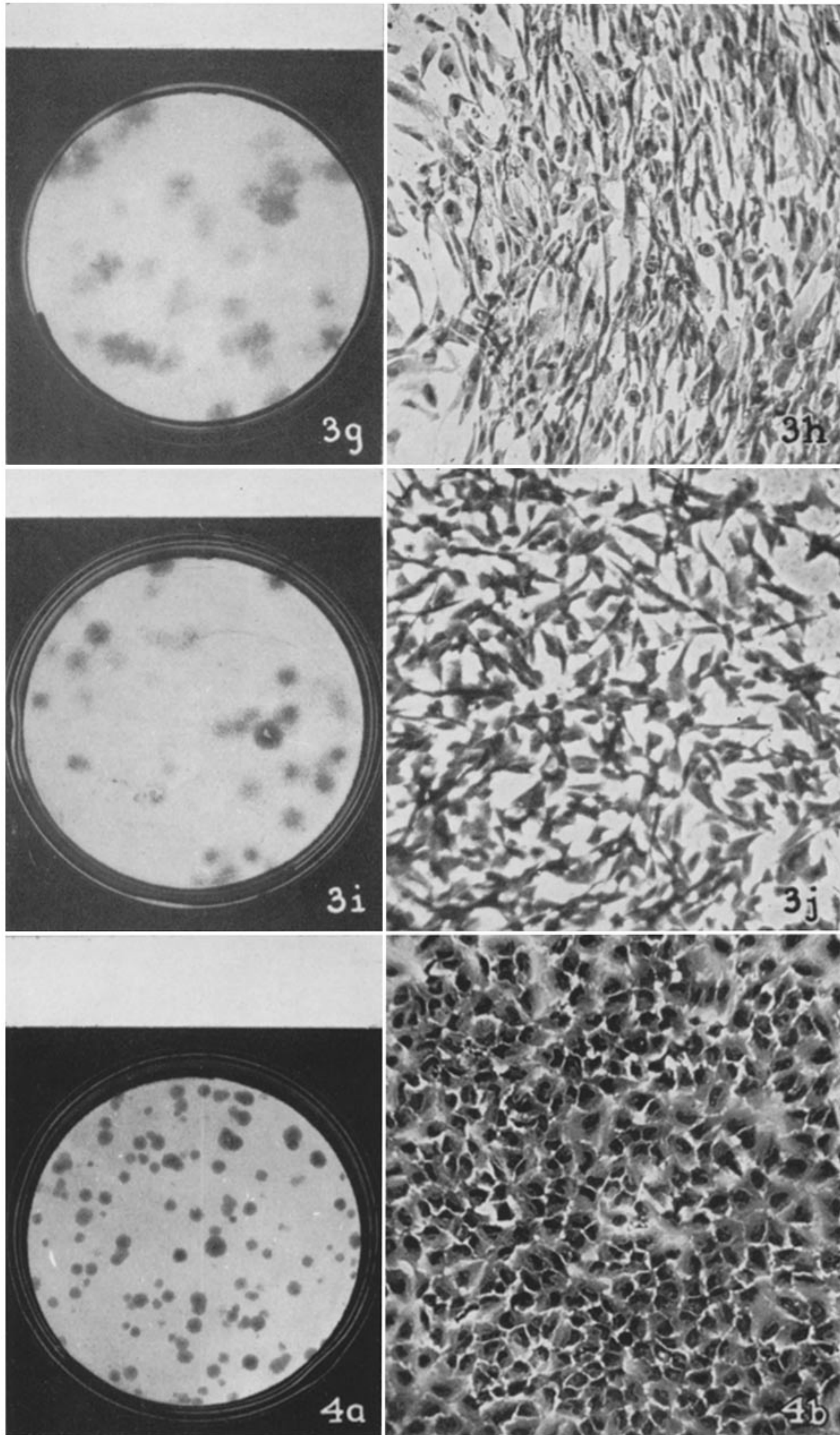
Fig. 3 *i*. Colonies of human amnion (Hoag) plated in "fibroblastic medium" without feeders. × 1.

Fig. 3 *j*. Enlargement of a portion of Fig. 3 *i*. × 100.

Fig. 4. Typical plating of single epithelioid cells, for contrast of colonial and cellular morphologies with the fibroblastic cells of Fig. 3.

Fig. 4 *a*. Colonies of human conjunctiva clone Cl plated without a feeder layer (2). × 1.

Fig. 4 *b*. Enlargement of a typical colony of 4 *a*. × 100.



(Puck *et al.*: Clonal growth of human fibroblastic cells)

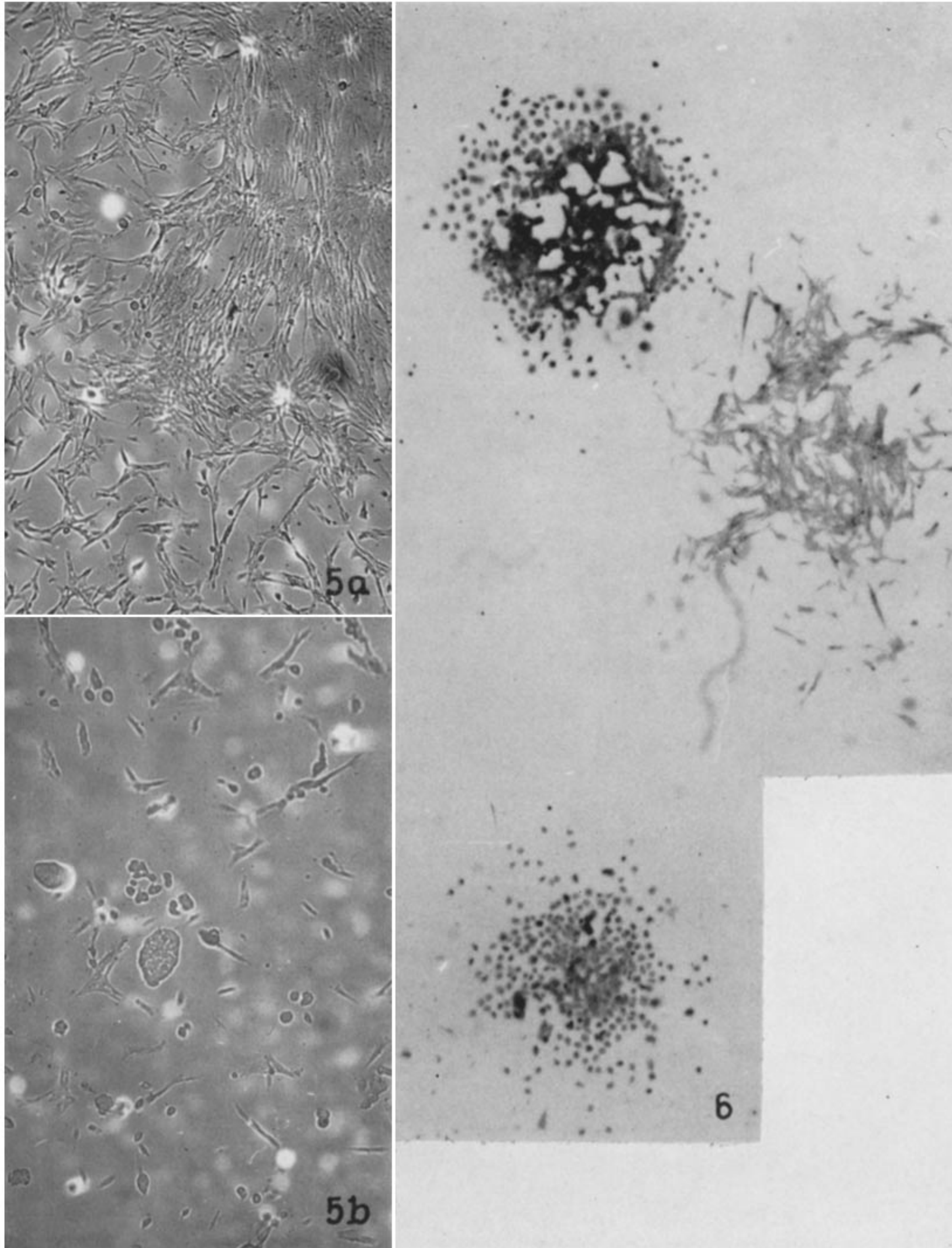
Plate 12

Fig. 5. Demonstration that inoculation of identical aliquots of a cell suspension obtained by direct trypsinization of a freshly taken biopsy of human lung produces growth which is completely fibroblastic in one medium and which contains many islands of epithelioid growth as well as occasional fibroblastic cells in the other.

Fig. 5 *a*. Typical field showing glass surface seeded with cell suspension from freshly excised human lung, in "fibroblastic medium" containing embryo extract. Heavy growth of motile, spindle-shaped fibroblasts occurs which rapidly covers the glass. × 40. This behavior would limit and obscure growth of any epithelioid cells contained in the original inoculum.

Fig. 5 *b*. Same cell suspension plated in "epithelioid medium." Growth of fibroblastic cells occurs but is sparse, so that the growth of epithelioid cells is revealed. Every field is studded with islands of tightly packed epithelioid cells. × 40. Such cells, when picked, breed true, reproducing the epithelioid morphology when plated in either medium.

Fig 6. Demonstration of clone isolation and stability of epithelioid and fibroblastic cell types from a bottle like that shown in Fig. 5, where single cells isolated from a human embryonic lung had been plated. A bottle like that of Fig. 5 was trypsinized, and aliquots of 50 to 200 single cells were plated again on Petri dishes in epithelioid medium. The photograph shows three typical colonies which developed on such a plate, two of which are typically epithelioid, and one fibroblastic. × 22. Such colonies, when picked and subcultured, continued to breed true to type.



(Puck *et al.*: Clonal growth of human fibroblastic cells)