CLONAL GROWTH OF MAMMALIAN CELLS IN VITRO

GROWTH CHARACTERISTICS OF COLONIES FROM SINGLE HELA CELLS WITH AND WITHOUT A "FEEDER" LAYER*

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PLATES 10 TO 12

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The conventional methods available for routine growth of animal cells *in vitro* demand the presence of a large cell population in order that sustained multiplication be initiated. This requirement for multicellularity has prevented application of many of the techniques developed for study of cellular growth, genetics, and genetic biochemistry in microorganisms, and, indeed, has raised the question whether somatic animal cells might not be fundamentally different from independent microorganisms like bacteria in the distribution of reproductive potential among the members of the cell population (1).

In an earlier paper of this series (2), we have described a method for growth of colonies from single HeLa cells, a strain of human epithelium cultured from a cervical carcinoma (3). The procedure is simple and rapid; it permits screening of large numbers of cells for genetic as well as physiologic studies; it is quantitative in that every cell of a HeLa population readily yields a colony, within an uncertainty no greater than sampling error. In the present communication, further methodological developments are presented and some of the characteristics of the resulting clonal populations are described.

Methods and Materials

This paper will be limited almost exclusively to consideration of the HeLa cell. The original cell population which was supplied to this laboratory by the George Washington Carver Foundation, will be referred to as the parental or wild-type strain.

The growth medium employed consisted of three components: a synthetic solution of nutrients, *i.e.*, sugar, amino acids, vitamins, and growth factors, in balanced saline (N),[1] mam-

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[1] The nutrient solution most commonly employed is that described by Dr. Charity Waymouth (4) and designated as A(50/2) with the exception that we have omitted Ca pantothenate and used only half the tyrosine called for in that formulation. Good results have also been obtained with other nutrient solutions, based on lactalbumin hydrolysate as the source of amino acids.

malian serum (S); and Hanks's balanced salt solution, which served as a diluent (D). The proportions of these three components adopted as a standard are: N, 40 per cent; S, 30 per cent; and D, 30 per cent. We shall refer to a solution of this composition as "complete growth medium." In all experiments in which it is not otherwise specified, the serum employed was a mixture of two parts pooled human[2] to one part horse. To all media penicillin and streptomycin were added as routine, each in concentrations of 100 γ/ml. When agar was employed, it was first washed (5). All glassware was washed by the procedures which have become standard in tissue culture. Incubation was performed, at 37°C. in fairly tight boxes, flushed continuously with a mixture of 5 per cent CO_2 in air, at a rate sufficient to maintain this gas concentration inside the incubator (about 1 complete gas change per hour). Trypsin (obtained from Nutritional Biochemicals Company, under the designation 1–300) was used in 0.25 per cent solution made up in Hanks's saline from which Ca, Mg, and PO_4 were omitted.

Definitions.—A *clone* is a population, all of whose members are descendants from the same single organism. *Plating efficiency* will be used to denote the per cent of cells set down as described below, which form colonies visible to the unaided eye within 8 days or less.

EXPERIMENTAL RESULTS

Growth of Clones with the Aid of Irradiated "Feeder" Cells.—

An earlier report (2) described how growth from single HeLa cells can be regularly accomplished by a plating procedure in which the test cells are placed over a layer of x-irradiated HeLa cells, these latter being unable themselves to multiply, but exhibiting active metabolism. Hence, these non-multiplying cells "condition" the medium so as to permit the single cells to reproduce to the point at which they eventually become self-sustaining. We have since found it simpler to use an earlier arrangement in which test cells and feeder cells coexist in the same layer, so that the extra manipulation involved in placing the test cells on a microscope slide resting on top of the "feeder" layer, is eliminated. The current "feeder" procedure is as follows: About 2×10^5 HeLa cells are pipetted on to the bottom of a 60 mm. Petri dish, in 4 cc. of complete growth medium. After a period of 5 to 18 hours during which the cells attach to the glass, the dish is x-irradiated with 4,000 to 5,000 r, a dose sufficient to ensure complete suppression of multiplication in all these cells (6). The medium is then removed and replaced with fresh medium containing the cells whose reproductive capacity is to be titrated. These cells find attachment space between the non-reproducing "feeder" cells, and proceed to grow into colonies during the subsequent incubation period. One medium change after 4 days of incubation usually suffices. As in the 2-layer method, the colony-forming efficiency of healthy HeLa cells is practically 100 per cent. Figs. 1 and 2 illustrate the resulting plates, and Fig. 7 presents a photomicrograph from a typical colony.

While the detailed x-irradiation survival curves of HeLa cells are being presented elsewhere, Fig. 1 demonstrates clearly that an exposure to 4000 r of a plate seeded with 2×10^5 viable HeLa cells does not leave a single survivor

[2] Pooled human serum was obtained from the George Washington Carver Foundation, Tuskegee Institute, Tuskegee.

capable of forming a macroscopic colony, even though such colonies readily develop if unirradiated cells are subsequently added, as shown in Fig. 2. The completeness of suppression of colony formation from such radiation exposures under the prescribed conditions has been confirmed in many repetitions of such experiments.

Growth of HeLa Clones without the Use of a "Feeder" Layer.—

In our earlier paper we pointed out that the success of the "feeder" principle in producing 100 per cent plating efficiency for HeLa cells proves that each cell possesses the potentiality for initiating unlimited growth, and implies that it is reasonable to expect similar results without a "feeder" system, provided proper conditions could be found. In the absence of specific knowledge of the chemical changes effected by the feeder cells, the desired objective might be attainable by improvement of the metabolic condition of the plated cells and by limiting diffusion processes after plating. The chances of success seemed good since we had demonstrated that deposition of as few as 11 HeLa cells in a microdrop regularly resulted in self-sustaining growth (2).

Study of a variety of factors (like lowering of the O_2 tension, which of itself was valueless) eventually led to realization that the standard trypsinization and washing procedure (5, 7) which had been used to disperse the cells subjects them to a considerable trauma that greatly impairs their ability to initiate growth. Other investigators (12) have also pointed out the necessity for gentleness during the trypsinization procedure. We have found that if this procedure is modified, so as to shorten the exposure time to trypsin, minimize mechanical stress, and eliminate all unnecessary washings, single HeLa cells can be regularly grown into large colonies by plating on Petri dishes in the absence of a "feeder" system. The following method for trypsinization and plating (which includes the use of agar when needed) has evolved into a standard procedure in this laboratory:

The nutrient medium is removed from the monolayer of HeLa cells grown to a confluent sheet in a bottle or Petri dish, and 0.25 per cent trypsin is added in amount sufficient to cover the cells to about 1 mm. in depth. The vessel is incubated at 37°C. for 15 minutes, with occasional gentle agitation, after which it is immediately cooled to room temperature. (One simple way to hasten this cooling and stop the trypsin action consists in addition of an equal volume of complete growth medium at room temperature at the end of the trypsinization period.) After a minimal period of agitation by pipetting, to complete dispersal of any clumps, the suspension is either diluted directly in growth medium for plating, or if washing is required by the conditions of the experiment the supernatant is replaced with fresh growth medium by means of a single centrifugation at room temperature for 5 minutes at a speed not exceeding 1,000 R.P.M. in an International centrifuge (model SBY No. 1). The absolute cell number is determined by counting with a hemocytometer. Any desired aliquot of this suspension (of which 90 per cent or more of the cells are single) is then transferred to a 60 mm. Petri dish containing 4.5 cc. of growth medium, and the plate is incubated in the CO_2 chamber. After about 5 to 15 hours, 0.6 cc. of a 1 per cent solution of washed agar made up in physiological saline may be added. Incubation continues for a total of 8 to 9 days. The purpose of the agar is twofold: By

increasing the viscosity of the medium, it decreases loss by diffusion of any necessary metabolites escaping from the cells. In addition, it depresses the tendency of individual cells to migrate which is exhibited by HeLa cells in certain media. Some strains of HeLa cells, when grown in the absence of human serum, form tightly adherent colonies, so that agar is unnecessary. (See Cell and Colonial Morphology below.) It is our feeling that agar aids growth of cells that are not multiplying optimally.

Trypsinization procedures (5, 7) which involve much cell agitation and many-fold washings result in extremely low colony-forming efficiency. With intermediate degrees of such cell manipulation, colony formation in the absence of "feeders" yields a plating efficiency in the neighborhood of 50 to 70 per cent. When maximal care is taken with trypsinization, the plating efficiency approaches 100 per cent. Thus, in a representative experiment in which 8 replicate platings each of 200 parental type HeLa cells were performed, the mean colony count was 198 and the standard deviation was 4 per cent of the mean. A typical plating is shown in Fig. 3 and a photomicrograph from a typical colony is presented in Fig. 8.

A possible clue to the nature of the trauma suffered by cells on repeated washing in saline which increases their dependence on a "feeder" cell system has been obtained in washing experiments carried out on a variety of animal cells grown in P^{32}-labelled medium. These experiments which are still progressing, show that a large fraction of the cellular P^{32}, including both large and small molecular constituents, is liberated from animal cells under these conditions. Such losses doubtless impair the cell's ability to initiate its reproductive cycle, even though no morphological damage is evident on microscopic examination. It is of interest that the amount of cellular P^{32} leaking out into the medium is much less when complete growth medium rather than Hanks's solution is used for washing.

The methods described in this and the preceding section have been successfully applied to clonal growth of several normal animal cells which have been found to give high plating efficiencies. The cell strains employed were isolated from normal human foreskin, conjunctiva, kidney, appendix, and liver, and from chick embryonic tissue. The details of growth and colonial development of such cells will be described in a subsequent paper, but it is worthy of notice here that of this group, the chick fibroblast alone has so far resisted cultivation as a clone except in the presence of a "feeder" system.

Colony Isolation and the Properties of Mutant Clonal Stocks.—

Colonies formed by single HeLa cells grown by either of the methods described have been isolated, picked, subcultured, and developed into standard clonal populations. The procedure is as follows: All but 0.5 cc. of the medium is removed and a sterile, stainless steel cylinder (6 mm. in diameter, 12 mm. high with 1 mm. wall) whose bottom edge has been coated with silicone stopcock grease (Dow Corning) is placed over the colony to be isolated. The silicone

forms a water-tight seal between the glass and metal. A few drops of trypsin solution are delivered into the open top of the cylinder and the plate incubated at 37°C. for 5 minutes. The supernatant liquid is sucked off slowly and nutrient medium replaced in the cylinder. Mild agitation then causes detachment of the cells from the glass, and the resulting suspension of clonal cells can be drawn off into a sterile syringe and transferred to a new vessel. As an alternative procedure, the single cells may originally be plated on a Petri dish, the bottom of which has first been coated with 1 cc. of 1 per cent agar made up in normal saline. After the agar solidifies, the cells are added in 4.0 cc. of growth medium plus 0.20 per cent agar. Under these conditions the cells grow into spherical colonies rather than two-dimensional monolayers. After 10 to 14 days of growth, these spheres can be isolated by means of a pipette and deposited in a new container. The clonal stocks are readily built up to populations of 10^7 in a single bottle and can be maintained indefinitely with unimpaired vigor through scores of passages. To insure genetic purity, each of our clonal stocks is passed through at least 2 single cell isolation procedures. We have designated the clonal strains of the Hela cell isolated here as S1, S2, S3, etc., (S for Dr. Florence Sabin).

Clonal stocks may be stored for periods of 1 month or more in stoppered tubes containing complete growth medium plus 0.2 per cent agar. These are inoculated with 200 to 500 cells, incubated for about 8 days at 37°C., and then transferred to room temperature (22°C.) for storage. The cells grow slowly under these conditions, developing into characteristic spherical clusters of about 1 mm. in diameter after 30 days.

Practically all the work in this laboratory is now carried on with pure clonal strains of HeLa cells. These stocks exhibit much greater uniformity of behavior than do those of the parental strain. Fig. 4 illustrates the colonial development in the absence of a "feeder" layer obtained from single cells of our S3 stock. The great uniformity of the resulting colonies stands in contrast to that obtained with the parental type grown under identical conditions[3] (*cf.* Fig. 3).

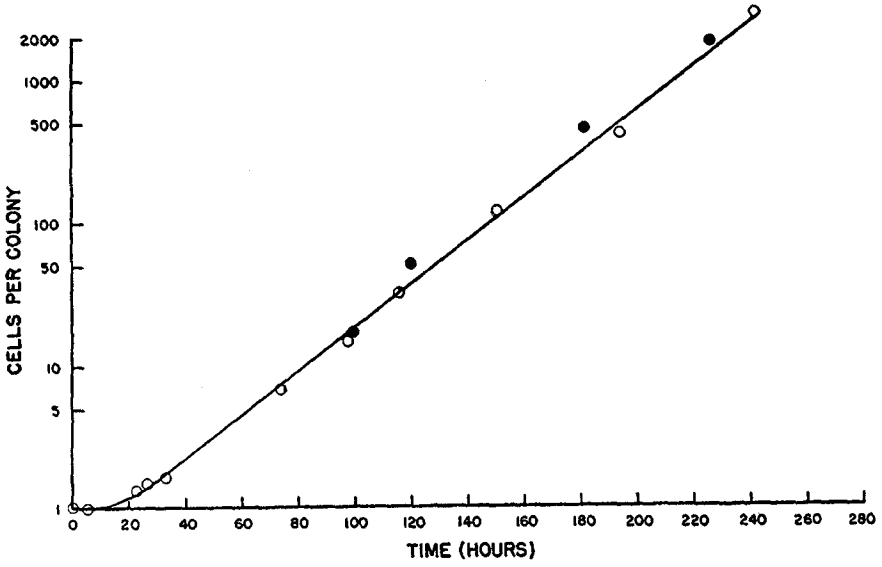
Different clonal strains of the HeLa tumor exhibit differences in characteristics like plating efficiency in media of certain compositions. These differences have persisted throughout more than 20 generations of growth and hence appear to be genuine mutational characters. Details of work with genetic markers will be presented in a further paper.

Growth Curves of a Clonal Population.—

The techniques here described make possible simple and accurate determination of the growth curve and division time of HeLa cells. Single cells are deposited in Petri dishes in nutrient medium and incubated in the standard fashion. At various time intervals microscopic count is made of the cell number in one or a few specific clones, or in an average of ten or twenty clones chosen

[3] The George Washington Carver Foundation, Tuskegee Institute has undertaken to distribute this clonal stock to interested laboratories.

at random on the plate.[4] The growth curves obtained are highly reproducible and exhibit an initial lag period of approximately 20 hours, followed by linearly logarithmic reproduction, as shown in Text-fig. 1. When the cell number exceeds 3,000, the slope may begin to flatten, doubtless because the two-dimensional character of the growth eventually results in crowding of the innermost cells. The curves obtained closely resemble those obtained for bacterial growth. In complete growth medium, our most active mutant strains exhibit a division



TEXT-FIG. 1. Typical growth curve of S3 strain of HeLa cells. The hollow and solid circles, respectively, represent two different experiments separated by an interval of 3 weeks. The extrapolated lag period is 18 hours and the generation time (time taken for the population to double) is 20 hours. As an index of the precision of the individual figures: Each point in the experiment represented by hollow circles is the mean of at least 6 and at most 43 colonies. The standard deviations of the cell counts averaged 22 per cent, and never exceeded 33 per cent.

time of 20 ± 2 hours, which is the maximal growth rate achieved by these cells under any conditions in our experience. Since the total lag period represents only one division time, and since 4 to 5 hours of the lag are required for the process of cell attachment to glass, the conditions of clonal growth in these plates appear to approach the optimum.

Cell and Colonial Morphology: Influence of Serum Factors on HeLa Cell Cohesiveness.—

It was noted that HeLa cells multiplying in the standard nutrient medium tend more often to form closely packed colonies in the presence of a "feeder"

[4] Plastic Petri dish covers with optically plane surfaces are particularly convenient, and are furnished by Plasticrafts, Inc., Denver.

system than in its absence. Similarly, the cells in a large colony are usually much more closely packed than in a small one. This aspect of colonial morphology is of considerable interest because of its possible relationship to factors which control the invasiveness of cancer cells (8). The availability of pure clonal stocks with their great morphologic uniformity is of particular convenience in the study of such a problem.

Experiments were performed with cells of the S3 clone. No "feeder" system was employed. Under these conditions it was found that the degree of cellular cohesiveness can be completely controlled by adjustment of the amount of human serum in the growth medium. The presence of pooled human serum to the extent of 10 per cent or more causes the cells to grow in a loose, highly extended meshwork. If human serum is omitted from the medium and replaced by bovine, porcine, or even a mixture of equine and bovine sera, the cells grow in an exceedingly dense, compact colony with polygonal array of cells, epithelial-like in appearance. These striking differences, evidence of wide phenotypic variation in a constant genotype, are portrayed macroscopically in Figs. 5 and 6, and microscopically in Figs. 9 and 10. The differences between the two types are evident throughout the entire colony in small colonies, and in large colonies are maximal for the cells at the edges because of the tendency previously noted for cells at the center of large colonies to assume a close packed form, even in human serum. It is of particular significance that, both in the presence or absence of human serum, the plating efficiency is 100 per cent and the growth rates are the same and maximal; *i.e.*, the generation time is 20 to 21 hours.

We conclude, then, that pooled human serum contains a factor which has no effect on the ability of the HeLa cell to reproduce, but markedly alters its cellular state from a highly compact to a loose, migratory structure.

Cells grown in the presence of 10 per cent or more of pooled human serum not only possess a distinct migratory property as shown by a comparison of the two different cell types of Figs. 9 and 10, but also expose much greater surface area. This was demonstrated by measurement of various cellular dimensions in an attempt to secure further information about the morphological differences produced by the two kinds of media. The cross-sectional area of each cell type was determined by microprojection on cross-sectional paper and planimetry of the area bounded by individual cells. Cross-sectional areas of the cell nuclei were also measured. Cell volumes were determined by microscopic measurement of the diameters of the spherical cells resulting from mild trypsinization. Living cells were employed in all these determinations, and measurements were repeated on at least 20 different cells of each type. The mean and standard deviation of each type of measurement is presented in Table I. They reveal clearly that individual cells grown in pooled human serum present four times as much surface area to the bathing medium as those grown in porcine or bovine serum. The surprising fact shown by the table is that,

despite this tremendous difference in cross-sectional area, both cell types have volumes which are identical within the limits of accuracy of the measurements. This constancy of volume, as well as the identity in the 2 cell types of other parameters, like nuclear cross-section, growth rate, and plating efficiency, suggests strongly that the basic cell change produced by the factor in human serum, has taken place in the surface membrane. In the presence of pooled human serum the cell wall appears to have a high affinity for glass and relatively little for neighboring cells, so that the structure is flattened and stretched, and tends to migrate away from its neighbors. In the absence of human serum, adjacent cell walls attract each other strongly, resulting in a compact structure of columnar cells with smaller cross-sectional area, but greater height.[5]

TABLE I

Measurement of Cell and Nuclear Cross-Sections, and Cell Volumes of Living Cells Grown (A) in the Presence of Pooled Human Serum and (B) in Non-Human Serum (Specifically Porcine)

The mean and standard deviation of each set of measurements (which included at least 20 and, in some cases, more than 200 cells) is given.

	A Growth in pooled human serum	B Growth in non-human serum
Cellular cross-sectional area.....	$1600 \pm 500\ \mu^2$	$370 \pm 100\ \mu^2$
Nuclear cross-sectional area.....	$200 \pm 45\ \mu^2$	$198 \pm 30\ \mu^2$
Cell volume.....	$(3.7 \pm 1.5) \times 10^3\ \mu^3$	$(5.0 \pm 1.9) \times 10^3\ \mu^3$

DISCUSSION

Growth outside the body of single animal cells into colonies has been described previously, as in the experiments of Moen with guinea pig fibroblasts (9) and Earle and his coworkers who sealed single mouse fibroblasts into capillaries (10). With both of these techniques only a small per cent of the cell inoculum yielded colonies, and it is impossible to determine whether this low yield is due to trauma suffered by the majority of the cells, or whether it expresses an intrinsic low multiplication potential of the cells from the particular tissues studied. The plating procedure described in this and a previous paper of this series makes possible simple and rapid titration of the number of multiplying cells in a population, and has demonstrated that for HeLa cells this figure

[5] The greater height of the cells grown in non-human serum was confirmed by phase microscopic examination of the colonies *in situ*. With each type of preparation, the microscope objective was first focused on the glass surface, then raised until the top of the living cell layer was in focus. The number of divisions on the calibrated fine adjustment screw was noted in each case. The cells grown in the absence of human serum were found to be approximately 4 times higher than the stretched variety, a value which is an excellent confirmation of the relationships shown in Table I.

is practically 100 per cent, and remains at this value throughout repeated subcultures. It becomes essential to determine how many different kinds of cells can be titrated by this technique, and what their plating efficiencies will be under different metabolic conditions.[6]

The plating procedure here described affords a simple and quantitative means of study of the action of agents which destroy cellular reproductive capacity. For example, the mean lethal dose of ionizing radiation for the reproductive function of a somatic mammalian cell has now been determined for the first time (6). In addition to measurement of absolute losses of growth function, it becomes possible to examine the effects of agents which change the generation time or the lag period with an accuracy and simplicity completely comparable to that available with a microorganism like *Escherichia coli.*

These methods also enlarge the scope of genetic investigations on mammalian somatic cells. The ability to establish clonal lines of mammalian cells rapidly is, of itself, only a small improvement on Earle's capillary technique (10). The real genetic contribution of the plating procedures here described is that it permits screening of huge populations so that the rare mutants can be isolated and developed into new strains. For example, if it were desired to isolate a mutant which occurs only once in a population of 10^7 individuals, it becomes necessary only to plate this number of cells in a medium which will support the growth of such a mutant but not of the wild type. No manipulation of the individual cells of the population is required. In this way, it becomes possible systematically to search for desired mutant characters, and to study quantitatively natural and artificially produced mutation rates, for stable genetic characters which are not susceptible to study by methods requiring special handling of each cell. Thus, the procedures developed in microbiological systems for study of gene biochemistry and neo-classical genetic processes (*e.g.*, transduction, transformation, conversion, cytoplasmic inheritance) would appear now to be capable of application to animal somatic cells.

These plating procedures also permit new studies of the action of animal viruses on their host cells. It now becomes possible to determine the effect of viruses on the reproductive capacity of an individual cell; to measure accurately how many virus particles are required to block this ability, reversibly or irreversibly; to investigate what changes of the virus particle are needed to prevent its exercising this action; and to test with confidence for the existence in animal cells of virus mechanisms similar to that of lysogenesis in bacteria.

The dynamics of the "feeder" system are of special interest. As Earle has pointed out, any necessity to "condition" a medium reflects only its lack of nutritional adequacy. In every case, therefore, once a cell strain can be induced to grow when nourished by a "feeder" layer, the next step in the process should

[6] Growth of single ascites tumor cells inoculated inside the living animal has been accomplished (11), a situation quite different from *in vitro* cell growth.

be to investigate what change in conditions will permit single cells to multiply in the absence of "feeders." However, the feeder principle is a powerful adjunct because it may permit the initial clonal cultivation of cells, after which it may be much easier to isolate clonal stocks and discover means for their adaptation to growth without "feeders." In principle, the feeder technique would appear to permit clonal cultivation of any cell for which large inocula can be made to multiply by standard tissue culture procedures.

The high metabolic activity of the "feeder" cells which enables them to condition the medium effectively despite their total loss of reproductive ability is understandable in view of the nature of the x-ray inactivating process. The ability of the cell to reproduce encompasses the duplication of the complete cell machinery, and hence is more vulnerable to damage by discrete, random, energetic events than is any of the more restricted cell functions. The total amount of energy absorbed by a cell irradiated with 4,000 r is $4{,}000 \times 2.03 \times 10^{-6}$ calories/gm., an amount of energy equivalent to a temperature rise of only 0.008°C. Hence the effect of the irradiation in multiplication must be confined to the production of a block in one or a very few of the metabolic chains necessary for growth.

It is particularly noteworthy that a factor exists in pooled human serum which can produce extraordinary effects on cell and colonial morphology, without appreciable change in the plating efficiency, growth rate, or cell volume, suggesting that the site of this action is localized in the cell wall. Coman and his coworkers (8) have demonstrated that cancerous epithelial cells differ from their normal counterparts in being deficient in the ability to bind Ca^{++}. This property prevents the invasive cells from adhering together, so that their natural ameboid motion results in extensive migration. Thus, the conditions provided by the presence of the responsible factors in human serum may, by alteration of the cell wall, play an important part in causing a localized tumor to become invasive. The nature of factors which can produce such a marked phenotypic change in mammalian cells of a constant genotype is of great interest.

Studies along the various lines which have been described are continuing.

SUMMARY

Two methods for simple and rapid plating of single HeLa cells, human, carcinomatous cells, are described. These result in growth and formation of colonies from each single cell. One of these procedures uses irradiated, non-multiplying "feeder" cells to condition the medium. The second requires more gentle handling of the cells, but otherwise is virtually the same as that used in plating bacteria on semisolid, nutrient media.

By extension of these methods, it is possible to isolate single mutant colonies and grow pure clonal stocks of animal cells. These genetically uniform strains are much more homogeneous in their behavior than the parental HeLa cell population.

Growth curves obtained from developing colonies are highly reproducible.

The most active mutant stocks so far isolated display a generation time of 18 to 20 hours.

In pooled human serum HeLa cells assume a highly stretched, ameboid form, with marked motility; whereas growth of the same cells in a variety of non-human sera results in tightly packed, columnar, epithelial-like morphology. The two cell types possess volumes, nuclear cross-sections, plating efficiencies, and generation times which are identical within experimental error, but display widely different cross-sectional areas, suggesting that the basic change occurs in the cell surface. It is conceivable that this change may be related to that which enables the cells of a compact tumor to become invasive.

Animal cells subjected to the standard trypsinization procedures which involve mechanical trauma and repeated washings in incomplete media leak large amounts of P and suffer impaired ability to reproduce as isolated cells.

Application of the methods described in this paper as a tool for quantitative study of normal mammalian cell growth, physiology, genetics, and biochemistry, and the response of cells to drugs, viruses, high energy radiation, and other agents have been indicated.

Grateful appreciation is expressed to the Colorado Serum Company, Denver, for unstinting aid in obtaining the various animal sera which were used in these studies.

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EXPLANATION OF PLATES

PLATE 10

FIGS. 1 and 2. Demonstration of operation of "feeder" cell system in growing colonies from single HeLa cells. Plates were incubated for 8 days, fixed with Bouin's solution, and stained with hematoxylin eosin.

FIG. 1. Plate with x-irradiated "feeder" cells alone. The granular background is due to the irradiated cells. Actual size.

FIG. 2. Identical plate as in Fig. 1, but which received in addition an aliquot of 100 normal cells. The colony count obtained is 97. Actual size.

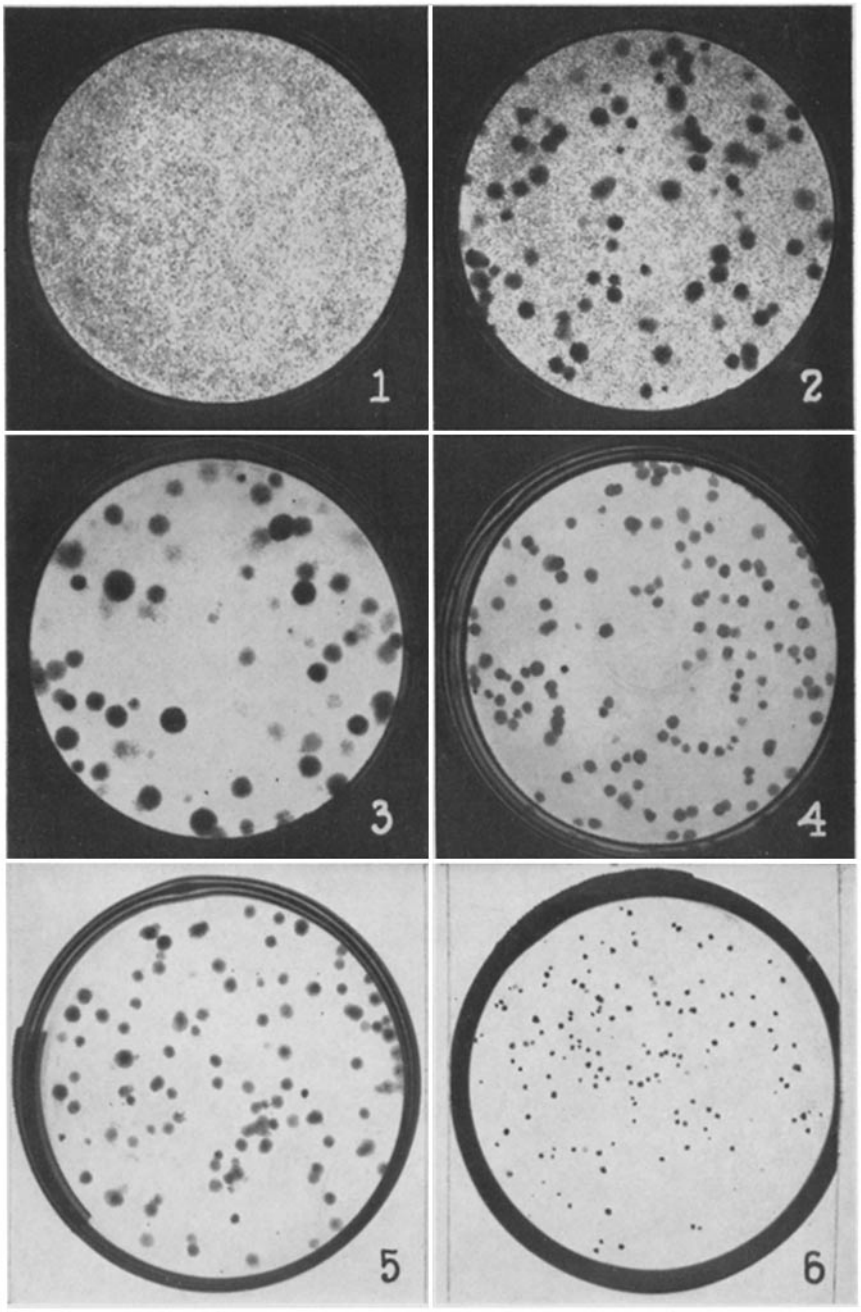
FIG. 3. Plate to which 100 gently trypsinized HeLa cells were added in an identical manner with that of Fig. 2, except that no "feeder" layer was employed. Actual size. These colonies generally show greater variation in density than do those grown over feeders (Figs. 1 and 2), tending to be somewhat more loosely packed.

FIG. 4. Colonies developed from single cells of a pure genetic strain (S3) of HeLa cells, under conditions identical with those used in the experiment shown in Fig. 3 (*i.e.*, no feeder system). The greater uniformity of the S3 cells as compared with the original HeLa population is obvious. The observed plating efficiency was 93 per cent. Actual size.

FIGS. 5 and 6. Effect of pooled human serum on the colonial morphology of S3 HeLa cells. Actual size.

FIG. 5. Complete growth medium, with human serum.

FIG. 6. Same, except that human serum was replaced by calf serum.

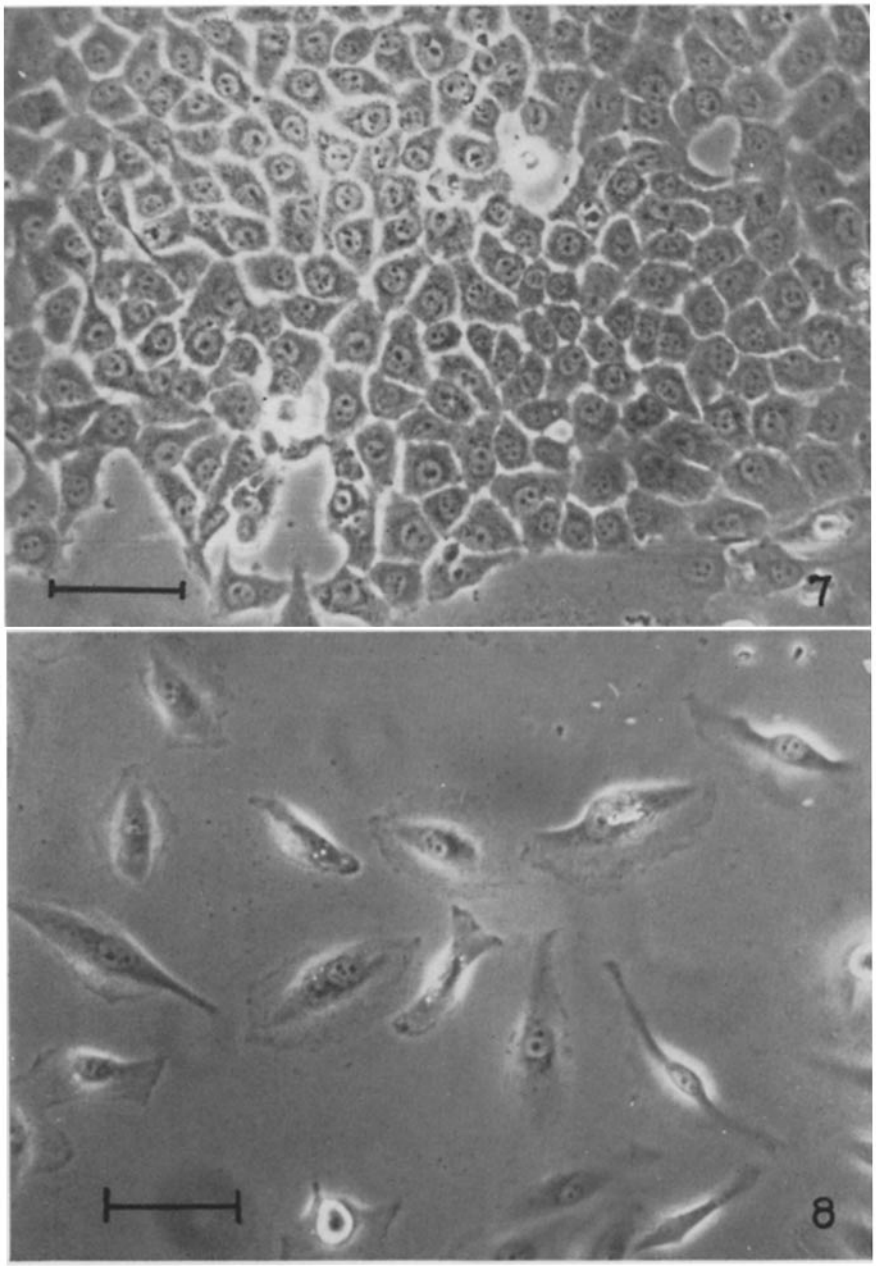


(Puck *et al.*: Clonal growth of mammalian cells *in vitro*)

PLATE 11

FIG. 7. Phase-contrast photomicrograph of cells at the edge of a HeLa colony (Fig. 2) which developed over a feeder layer. Colonies of such cells generally are fairly close packed, although more diffuse colonies can also be found. The black line represents a distance of 100 μ.

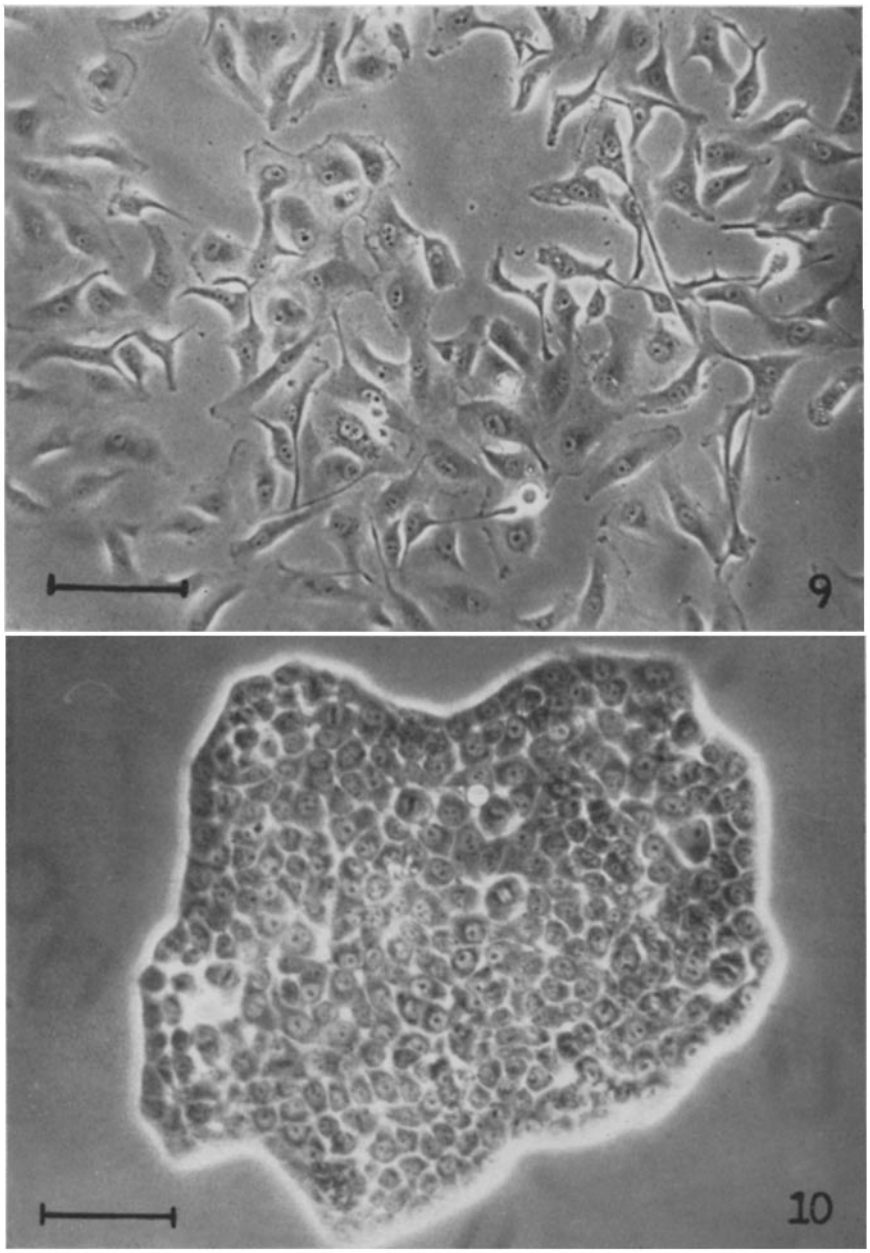
FIG. 8. Phase-contrast photomicrograph of cells in one of the most loosely packed colonies shown in Fig. 3. The cell density in the colonies from parental type HeLa cells developing without feeders, in complete growth medium (containing human serum) varies between this extreme and that shown in Fig. 7. The black line represents a distance of 100 μ.



(Puck *et al.*: Clonal growth of mammalian cells *in vitro*)

PLATE 12

FIGS. 9 and 10. Photomicrographs of typical colonies growing in the presence and absence of human serum. In the presence of the human serum, the cells are long and stretched, and very diffusely spread (Fig. 9), while in its absence, they are cuboidal and extremely closely packed (Fig. 10). All conditions were identical in the two platings. The black line represents a distance of 100 μ. (Phase contrast.)



(Puck *et al.*: Clonal growth of mammalian cells *in vitro*)