GENETICS OF SOMATIC MAMMALIAN CELLS

I. Demonstration of the Existence of Mutants with Different Growth Requirements in a Human Cancer Cell Strain (HeLa)*

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Plate 31

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In previous communications (1, 2) methods have been described for growth of single mammalian cells into colonies of any desired size. These methods are simple and rapid, and have been applied to cells originating from both cancerous and normal tissues (2, 3). Plating efficiencies (*i.e.* the proportion of the single cells plated which yield macroscopic colonies) close to 100 per cent have been readily obtained with a variety of human cells. These techniques make possible study of the mammalian somatic cell as a microorganism and promise to permit bridging of at least some of the gaps between genetic operations hitherto confined to microorganisms on the one hand, and multicellular forms on the other.

Study of such genetic processes requires methods for examination of the hereditary behavior of individual cells, and the isolation of mutants with deviant metabolic characteristics. Such operations are extremely difficult with techniques involving only many celled populations, because of uncertainties regarding the genetic purity of the starting population, and the degree to which a given characteristic may depend on the interaction of two or more cells. Micromanipulative techniques with individual cells become laboriously prohibitive for studies requiring screening of huge numbers of cells in order to isolate rare mutants. When large numbers of single cells can be grown with high efficiency into macroscopic colonies in a single plating operation, the isolation and identification of mutants becomes greatly simplified and analogous to the corresponding procedure in bacteriology (4).

The present paper describes application of the single cell plating procedure to the isolation of mutants in the population constituting the HeLa strain—a human, cervical carcinoma first isolated by Gey and his associates (5) and maintained in tissue culture for a period of several years. The characterization of two stable mutants with different growth requirements is described. In addition, further observations on the operation of a "feeder" system of x-rayed

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cells on the growth of single cells are presented, and their implications discussed.

Definitions.—The terms *clone* and *plating efficiency* are used as previously defined (2). *Mutation*—any change in heredity not ascribable to sexual segregation and recombination (6). (See Discussion.) *Parental population*—a cell strain arising directly from material obtained by a macroscopic biopsy, with or without subsequent cultivation in tissue culture, but which has not been developed as a clone from a single cell, *in vitro*. *Feeder layer*—a layer of cells x-rayed with at least 2000 r, which exhibits no multiplication itself, but supports growth of an inoculum of single cells deposited over it (2, 7). *Average generation time*—an index of the rate of multiplication of single cells, obtained by dividing the incubation time in hours by the number of generations represented by the average number of cells per colony. This calculation ignores the effect of lag periods. But since these are almost always less than 24 hours in our experience (2) and the incubation periods employed are never less than 9 days, we use this approximate figure instead of the actual generation time which requires construction of a complete growth curve, except in experiments demanding maximal preciseness.

Methods and Materials

The methods employed are described in detail in previous publications (2). In all the experiments described here, the medium employed for cell growth contained 3 components: our standard solution of salts, glucose, amino acids, vitamins, and growth factors (2), which was always present in a fixed concentration of 40 per cent; mammalian serum—human, animal, or a mixture of both—present in variable concentration which is always specified; and Hanks's saline, added in quantity to make the total 100 per cent. Since, for all the present experiments, the serum component is the only significant variable, the medium employed in each case will be identified by listing its serum composition. Human serum was obtained from healthy, young, adult donors. Animal serum was obtained from fasted, healthy horses or pigs.

The use of a "feeder" system involves plating the single cells which are to be grown into colonies on a Petri dish already seeded with a layer of x-irradiated cells, which themselves do not multiply. These non-reproducing cells can change the environmental medium so as to permit colony formation by subsequently added viable cells which otherwise might not reproduce (1, 2). Approximately 10^5 HeLa cells are pipetted into a 60 mm. Petri dish in 4.0 cc. of the growth medium to be tested. After a period of incubation which can vary from 2 to 18 hours, the dish is irradiated with 4000 r, a dose sufficient to insure complete suppression of sustained multiplication in the feeder layer (7). The inoculum of cells whose reproductive capacity is under test is then added and the plates are incubated. The medium may require replacement after 6 to 7 days.

EXPERIMENTAL RESULTS

It was earlier shown (2) that when cells of the parental HeLa population are singly plated on top of a feeder layer, the plating efficiency approximates 100 per cent, yielding colonies which are dense and fairly uniform in appearance. In the absence of a feeder system, however, colonial morphologies are more heterogeneous and, although the plating efficiency still approximates 100 per

cent, an appreciable fraction of the colonies which develop have quite small cell numbers. It was also demonstrated that when one particular clone had been picked and subcultured, its single cells regularly produced large, densely staining colonies of much greater uniformity than those of the parental population. (*Cf.* Figs. 3 and 4 of Plate 10 in reference (2)). These observations suggested that the parental population consists of individuals with different, hereditable growth characteristics.

From plates containing colonies grown from single cells of the parental population, four different clones were selected and isolated for subculture by means of the steel cylinder technique described earlier (2). This report compares the properties of two of these clonal stocks, respectively designated S1 and S3, which were isolated from the parental Hela cell population.

Growth in Different Serum Concentrations without a Feeder System.—

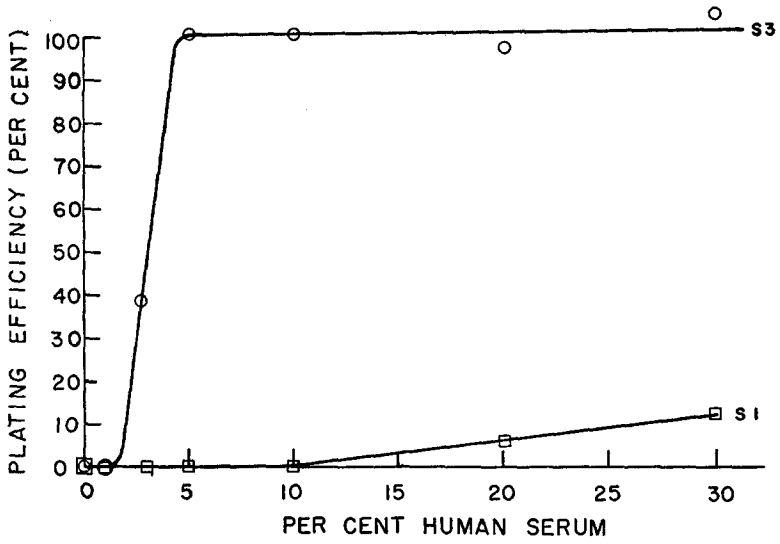
Both S1 and S3 cell strains have the same typical HeLa cell morphology, and both readily display the ability to grow either in tightly packed, columnar form, or as loose, migratory, stretched cells, depending on whether the serum component is equine, bovine, or porcine, on the one hand, or human on the other (2).

Studies of the plating efficiency and average growth rate of the two cell types in various serum concentrations were carried out. Such experiments revealed that S1 has a much lower plating efficiency than S3 in any concentration of human serum employed. At concentrations of human serum between 5 and 10 per cent, the difference in behavior of the two clonal strains becomes maximal; *i.e.*, the plating efficiency of S3 is 100 per cent, while that of S1 is practically zero. These relationships are illustrated in Text-Fig. 1.

Even those colonies of S1 which do form in the presence of 20 to 30 per cent human serum contain many fewer cells than S3 grown under identical conditions. Thus, the average number of cells per colony formed after 9 days' incubation of S1 in 30 per cent human serum was 96, and in 20 per cent serum the value was only 60 cells per colony. In contrast, S3 colonies averaged 1300 cells in 20 or 30 per cent serum. Even in 5 per cent serum, in which S1 does not grow at all, S3 colonies contained an average of 300 cells after 9 days' growth, a value equivalent to an average generation time of 26 hours, and only slightly longer than the optimal reproductive time for S3. Figs. 1 a to 1 d illustrate typical platings of each cell type in two different serum concentrations.

When an animal serum like equine or porcine is used instead of human serum the general pattern of events is the same, except that the growth of S1 tends to be somewhat better in high concentrations of animal serum than it is in human. Thus, in 20 per cent horse serum S1 may occasionally exhibit a plating efficiency as high as 60 per cent. However, the number of cells per colony still tends to be low. Moreover, as the animal serum concentration is diminished, the difference in behavior of S3

and S1 becomes just as great as with human serum. In general, concentrations in the neighborhood of 2 to 6 per cent of horse or porcine serum usually act like human serum in producing 100 and 0 per cent plating efficiencies of S3 and S1, respectively. In many experiments, the serum component employed was a mixture of 2 parts human to 1 part horse serum, which regularly gives clean separation of the two strains.



TEXT-FIG. 1. Typical experiment demonstrating the change of plating efficiency of the S1 and S3 clones with change in concentration of human serum in a medium constant in the composition of other components. The range of serum concentration at which maximum difference in behavior of the 2 strains occurs varies somewhat with different sera, but usually encompasses the interval of 2 to 10 per cent. Horse or porcine sera behave similarly, though with a slightly narrower concentration range for optimal differentiation. A mixture of 2 parts human to 1 part horse serum can also be employed to differentiate the two strains, with excellent results.

Stability of the Genetic Trait.—

Experiments were performed to test whether the differing growth response to reduced serum concentration of these two clonal strains is a trait sufficiently stable to serve as a useful marker for genetic studies. Both cell strains have been grown as routine in bottles, using massive inocula for transfer during the 5 months after their first isolation from single cells. During this time no change in any characteristic of either population had been observed, and the differential action of low serum concentrations had been established. As a more rigorous test of the stability of the genetic trait under study further single cell isolations were performed. Two new clonal isolations were made from S1 and one from S3. These were subcultured, regrown, and tested, and found in every case to duplicate the behavior of the parental clonal stock from which they had orig-

inated. When a single cell is grown up in this way to a population of 10^7, approximately 23 generations have elapsed, so that the test of genetic stability is equivalent to a large number of serial transfers of large inocula in bottles. Each of these cell strains has been in continuous cultivation for 13 months in this laboratory and has passed through a total of approximately 100 successive generations, including two single cell isolations. During this period, each strain has maintained its original behavior with no recognizable alterations.

Effect of a Feeder System.—

The foregoing experiments pose a problem in explaining the persistence of the S1 genotype in the parental HeLa population, which has been maintained for years by cultivation in human serum (8). On the basis of the data here presented, one would have expected S3 to overgrow and displace S1 completely in a period of perhaps 100 to 200 generations. Since this has not occurred, and since the difference between the S1 and S3 traits is apparently stable genetically, the operation of some compensating mechanism is suggested which, in tissue culture procedures, neutralizes the selective advantage displayed by S3 when grown as isolated single cells.

It seemed likely that the persistence of S1 in tissue cultures maintained by means of large inocula might be an expression of cell cooperation whereby a close association with other cells might improve the growth of S1. The use of feeder systems readily lends itself to exploration of this possibility by test of the growth of S1 in a deficient medium supplemented with a feeder layer of metabolizing, but non-reproducing, irradiated cells. Experiments were carried out in which S1 was plated with a feeder layer of x-rayed S3 cells, in a medium whose serum composition produces no growth of S1 whatever in the absence of feeders. The presence of the feeder cells raised the plating efficiency of S1 from 0 to almost 100 per cent. In addition, the growth rate achieved was comparable to the optimal exhibited by S3. Thus in a typical experiment in which the total serum concentration was 10 per cent, the average colony size after 10 days' incubation was 500, a value equivalent to an average generation time of 27 hours, and very close to that exhibited by S3 in the same medium. It follows that the presence of the feeder system produced optimal growth of S1 in a medium which yielded no growth in its absence, and so eliminated the selective advantage exhibited by S3 in the absence of feeders. Figs. 2 a and 2 b present photographs demonstrating the growth obtained under these conditions.

DISCUSSION

The foregoing experiments demonstrate that the parental HeLa population, while morphologically uniform, contains a mixture of genetically stable cells differing in their growth requirements. Of the two types studied, only one can

grow with 100 per cent plating efficiency in the absence of a feeder system, in any of the media so far employed. These studies demonstrate that the genetic purity of a cell strain cannot be taken for granted on the basis either of its morphologic purity, or of having grown for a period of time, however long, in tissue culture by procedures involving massive inocula.

The availability of alternative growth conditions which result in plating efficiencies for the deficient strain in the neighborhood of 100 or 0 per cent, respectively, makes possible quantitative determination of each cell type in their mixtures. Experiments using this technique are in progress to determine whether high energy irradiation can effect mutation of these two types of growth response.

The present experiments give no information on the mode of origin of the differences between S1 and S3. All the cells from a given mammalian individual arose initially from the same fertilized egg, and so, at least in a formal sense, clonal cell lines of divergent genetic constitution isolated from a mammalian individual directly or indirectly, may be called mutants. Such a designation contains no implications concerning the mechanism by which such changes have arisen, their frequency, degree of reversibility, or randomness. The notation is convenient and in accord with the operational definition current in microbiological genetics. Once such a mutation has been demonstrated, the question must next be attacked as to whether the observed change involves nuclear or other genetic determinants; whether viruses, transforming principles, or related agents may be operative; or whether some complex mechanism which chooses for expression one of a variety of possible latent hereditary characters, as has been demonstrated in *Paramecium* (9), may be involved.

The presence in a cell population of diverse mutants, one or more of which may find a selective advantage as a result of a changed metabolic situation, and so outgrow other cells, has been invoked to explain changes in cell characteristics related to invasiveness (10). If such mutant cells can be readily characterized, methods like those described here should prove valuable in cellular analysis of the population dynamics of such systems. Single cell plating should permit identification of situations in which large numbers of cells are suddenly changed in their behavior, as opposed to gradual change due to shift in the proportion of mutants present in an ininitially heterogenous population. It would be of interest to study by these methods situations demonstrating gradual progression to the carcinomatous state (11).

The cooperative action between S1 and S3 mutants demonstrated by the present experiments with a feeder system raises many possibilities for exploring cell-cell interaction between similar, as well as different, cell types. These results establish that a feeder system can overcome a genetically controlled deficiency for growth in a particular medium. Further studies with this technique are under way, testing the abilities of different feeder systems to in-

fluence various cell operations. This approach may permit study of a variety of phenomena in addition to multiplication, wherein one cell supplies another with the conditions necessary for particular functions. Experiments are also planned to identify the serum constituents which are capable of differentiating S1 from S3 and which become unnecessary in the presence of a feeder system.

SUMMARY

The parental HeLa cell population, a morphologically uniform, human cancer cell strain, grown for several years in tissue culture by procedures always involving massive inocula, has been shown to contain different mutant cell types.

Two clonal lines have been isolated and established as reliable stock cultures. Both strains exhibit 100 per cent plating efficiency in high or low serum concentrations in the presence of a feeder system. In the absence of a feeder system and in low serum concentrations, the two strains are quantitatively differentiable: S3 still exhibits 100 per cent plating efficiency, while that of S1 lies in the neighborhood of zero.

These differences have remained stable throughout 100 successive generations of growth of each strain including 2 single cell isolations.

Application of these techniques to studies in the genetics of mammalian somatic cells and to specific cell-cell interactions has been indicated.

BIBLIOGRAPHY

1. Puck, T. T. and Marcus, P. I., *Proc. Nat. Acad. Sc.*, 1955, **41,** 432.
2. Puck, T. T., Marcus, P. I., and Cieciura, S. J., *J. Exp. Med.*, 1956, **103,** 273.
3. Marcus, P. I., Cieciura, S. J., and Puck, T. T., J. Exp. Med., in press.
4. Lederburg, J., Microbial Genetics, Madison, University of Wisconsin Press, 1951.
5. Gey, G. O., Coffman, W. D., and Kubicek, M. T., *Cancer Research*, 1952, **12,** 264.
6. Darlington, C. D. and Mather, K., The Elements of Genetics, New York, The Macmillan Co., 1950, 405.
7. Puck, T. T., and Marcus, P. I., *J. Exp. Med.*, 1956, **103,** 653.
8. Scherer, W. F., Syverton, J. T., and Gey, G. O., *J. Exp. Med.*, 1953, **97,** 695.
9. Sonneborn, T. M., *Harvey Lecture*, 1950, **44,** 145.
10. Klein, E., *Exp. Cell Research* 1955, **8,** 188.
11. Rous, P. and Beard, J. W., *J. Exp. Med.*, 1935, **62,** 523.

EXPLANATION OF PLATE 31

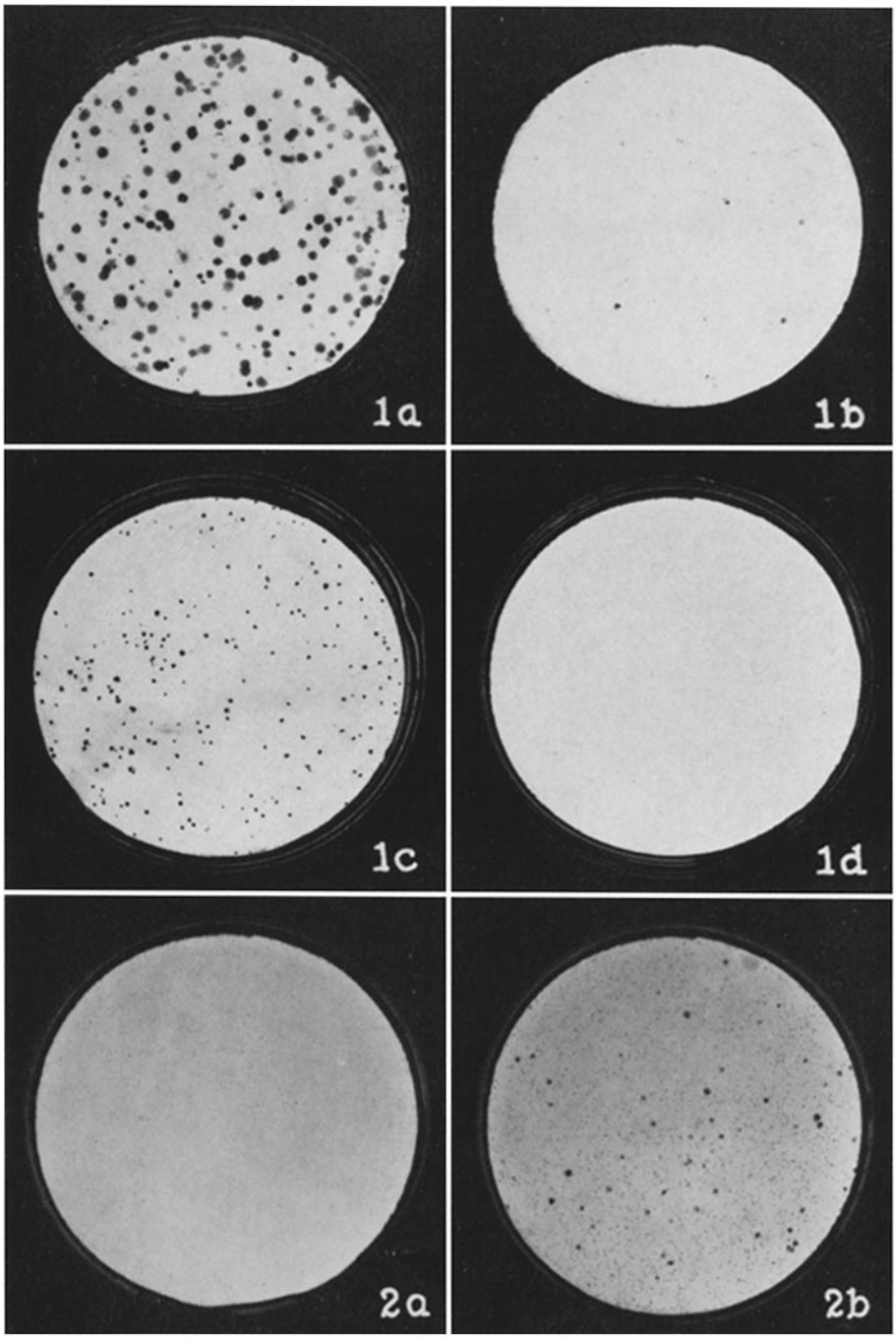
FIGS. 1 a to 1 b. Differential growth of S3 and S1 in different serum concentrations. 200 cells were inoculated on each plate as follows:—

Fig. 1 a. S3 in 15.0 per cent serum; Fig. 1 b. S1 in 15.0 per cent serum; Fig. 1 c. S3 in 2.5 per cent serum; Fig. 1 d. S1 in 2.5 per cent serum.

The lower serum concentration produced clearest differentiation between the two strains. The serum used in these experiments consisted of a mixture of 2 parts human to 1 part horse. All photographs are actual size.

FIGS. 2 a and 2 b. Demonstration that a feeder system raises the plating efficiency and growth rate of S1 to values approximating that of S3. Each plate received an inoculum of 100 S1 cells, and was incubated for 10 days.

Fig. 2 a. 3 per cent serum, no feeders. No growth has occurred in 10 days. (Actual size.) Fig. 2 b. 3 per cent serum plus a feeder system. The colonies are clearly seen as the densely staining areas, which stand out against the speckled grey background of giant feeder cells (7). The plating efficiency on such plates averaged 84 per cent. Note that the colony size is similar to that of the S3 colonies shown in *c* of Fig. 1, which were grown in approximately the same serum concentration for almost the same period of time (9 days as compared with 10). (Actual size.)



(Puck and Fisher: Genetics of somatic mammalian cells. I)