CULTIVATION OF CELLS IN VITRO AS ISOLATED COLONIES

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The cultivation of cells as isolated colonies, a technique developed in Puck's laboratory [12, 14], has been extensively applied to the genetics of the somatic cells of mammals and man and has contributed to the rapid development of this subject [1-4, 6, 12]. The basic condition of success in the development of this technique has been the use of balanced synthetic nutrient media in conjunction with animal sera [12, 13, 15]. However, attempts to reproduce the technique have encountered several difficulties of a technical nature.

In the present paper we describe the results of experiments to cultivate cells in vitro as isolated colonies. Our own technique differed slightly from Puck's (separation of cells by means of versene, cultivation in Carrel flasks, the use of a different synthetic medium, and so on). Our efforts were directed towards obtaining maximal efficiency of seeding [13], i.e., the maximal number of colonies developing from the seeded cells.

EXPERIMENTAL METHOD

The investigation was conducted on a monolayer culture of human HeLa cells. The following lines of this strain were used: 1) the initial parent strain HeLa*; 2) the clone line HeLa† [6]; 3) the clone line K_5-1, isolated by Puck's method [13]; and the subclones K_5-3 and K_5-8, obtained by E. P. Ugryumov [5]. Passage of the cultures took place every 7 days in Carrel flasks, 6-7 cm in diameter, when from 300,000 to 500,000 cells taken from the plate with versene were seeded. The complete nutrient medium contained 90% of medium No. 199 and 10% of ox serum.

The technical factors common to all the experiments to cultivate cells as isolated colonies were as follows. Cultivation took place in the same Carrel flasks on a nutrient medium composed of medium No. 199 and ox serum. Seven-day cultures were used. Immediately after removal of the medium the layer of cells in the flask was covered with warm versene solution and the flasks were incubated for 10-15 min. The cells separated from the glass were precipitated by centrifugation (500 rpm) for 10 min, and then suspended in complete nutrient medium. The cells were counted in a Goryaev's chamber, living and dead cells being counted separately. Dead cells were stained with a 1% solution of trypan blue. Serial dilutions were then made in order to obtain suspensions of the required concentration. The necessary number of cells in 10 ml of complete medium by volume were then seeded into Carrel flasks, which were incubated at 36°. After the end of incubation the medium was decanted, the flasks washed out once with Hanks's solution, and the colonies on the glass were fixed with Bouin's fluid and stained by Giemsa's method or methylene blue. The experimental results were assessed by the number and size of the colonies. The size has not usually been given in the literature. Our experiments showed that measurement of the size of the colonies gives additional valuable information. It was determined by measuring the diameter of the colony with an ocular micrometer through a binocular loupe, and was expressed in scale units (ocular 8×, objective 1×).

EXPERIMENTAL RESULTS

In the preliminary experiments cultures of the parent line and of the clone lines HeLa K_5 and K_5-1 were used. The first experiments showed that HeLa cells taken from the glass with versene and seeded in small doses into Carrel flasks can give rise to growth of colonies varying in number, size, and morphology.

* Obtained from the Institute of Virus Preparations (Moscow) in December, 1959.
† Obtained from the Institute of Experimental and Clinical Oncology of the USSR Academy of Medical Sciences in December, 1959.

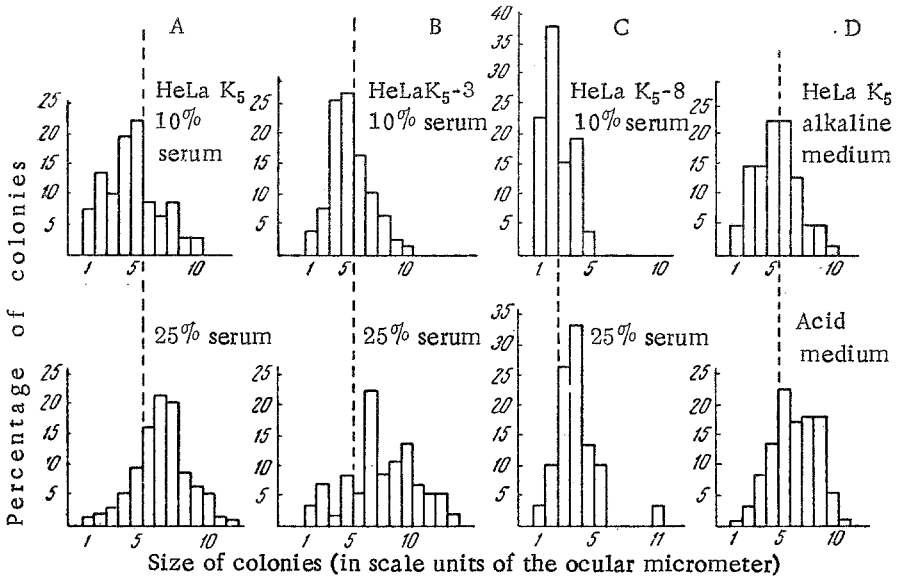

Fig. 1. Size of individual colonies in experiments to study the effect of the concentration of serum in the medium (A, B, C) and the pH of the medium (D). Each diagram shows the distribution of colonies by size in one of the flasks. The total number of colonies for each flask is taken as 100%.

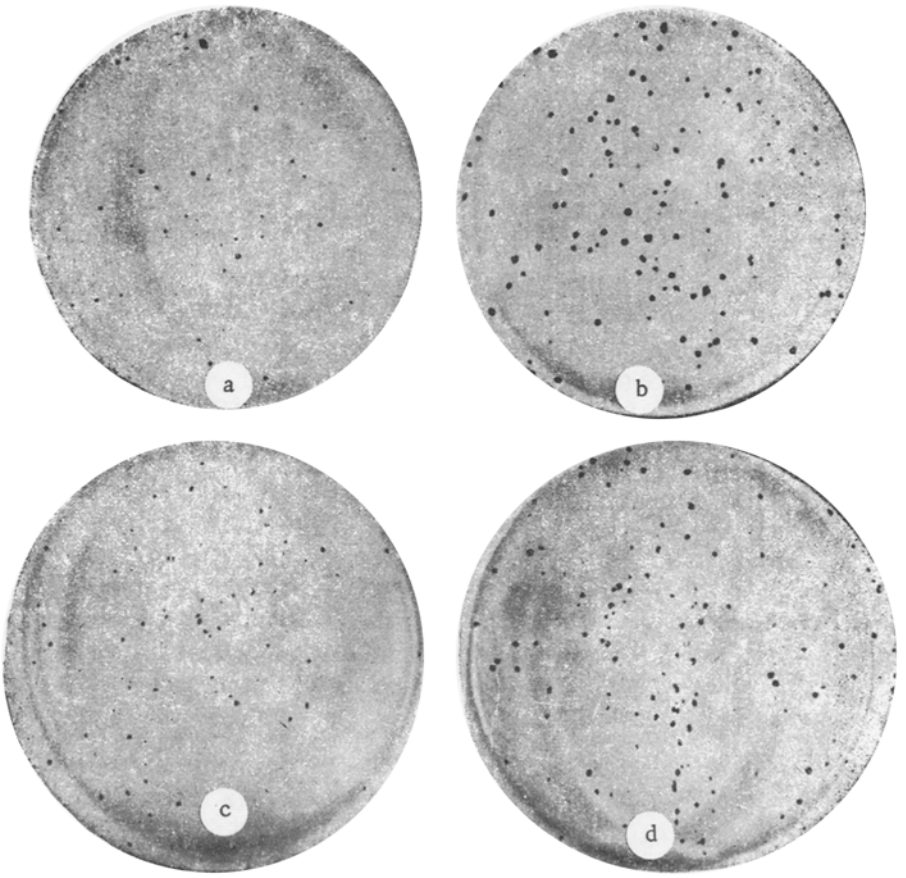

Fig. 2. Colonies of cells of different lines of strain HeLa K_5 in media containing 10 and 25% of serum (a, b) and in media with a weak alkaline or weak acid pH (c, d).

The number of cells seeded significantly affected the development of the colonies. When numerous (1000 or, in particular, 10,000), the overwhelming majority of cells formed colonies, whereas if 100 cells were seeded none were formed. The number of cells also had a marked effect on the rate of development of the colonies. With a large dose, the colonies could be distinguished clearly with the naked eye during the first days, whereas after seeding 100 cells, colonies of the same size appeared much later.

The pattern of development of the colonies changed considerably depending on the duration of cultivation. With the naked eye, colonies could be distinguished in the first week after seeding, but at the end of the second week they had become large (Figs. 1 and 2). During subsequent cultivation the separate colonies merged and underwent degenerative disintegration with detachment of the central areas of the larges colonies, and the appearance of new, small colonies, presumably as a result of secondary seeding of the cells. We concluded from these observations that the optimal number of cells during seeding in order to produce growth in the form of colonies lay between 100 and 1000 cells, and that cultivation should continue for not less than 1 and not more than 2 weeks. In all subsequent experiments with HeLa cells we used a dose of 200 cells for seeding and cultivated for 2 weeks.

The seeding efficiency and, in particular, the morphology of the colonies differed from one line to another, even within the limits of the one strain of HeLa which we used. The difference in the character of growth (epithelioid or fibroblast-like growth) was clearly reflected both in the morphology and in the size of the colonies developing from one cell. Epithelioid cells formed compact colonies, round in shape, with clear, smooth edges. The fibroblast-like cells characteristically produced larger, friable colonies, irregularly circular in shape, with uneven edges. Between these extreme types of colonies intermediate forms were usually seen.

Because of the low efficiency of seeding in the first experiments and the considerable variation in the number and size of the colonies, particular attention was directed towards perfecting the technique of preparation of the cell suspension so that it should consist of separate viable cells. Suspensions were used for the experiments in which single living cells constituted not less than 90% of the total. Two serial dilutions of the original suspension were made in order to obtain concentrations of 20,000 and 2000 cells in 1 ml. From the last dilution (containing 200 cells in 0.1 ml) a suspension was prepared containing 200 cells in 10 ml of medium, and this was poured into flasks.

In the principal experiments clone lines HeLa K_5, K_5-3, and K_5-8 were used. Experiments taking account of all these various technical factors were carried out in order to study the effect of the medium on the development of colonies. The results of the experiments to grow colonies on a medium containing different concentrations of serum are given in Table 1 and in Figs. 1 and 2. The experiments with clone K_5 were carried out separately from those with clones K_5-3 and K_5-8, and the latter were always carried out in parallel series. The experimental results are summarized in Table 1, which shows that an increase in the serum concentration by 250% over the usual level gave a considerable increase in the number of colonies in all the experiments with clone K_5 and in some of the experiments with subclones K_5-3 and K_5-8. The number of colonies from K_5 cells was twice as great in medium containing 25% serum, and reached 72% of the number of seeded cells. The effect of serum was still more demonstrative if the colony size was analyzed. The colonies were significantly larger in medium containing 25% serum, even when no effect of the serum on the number of colonies could be detected (clones K_5-3 and K_5-8). Analysis of the percentage distribution of

TABLE 1. Development of Colonies of Cells of HeLa Lines K_5-3 and K_5-8 in Media with Different Concentrations of Ox Serum

Experiment No.	Line of strain	10% serum			25% serum		
		No. of colonies in 1 flask		mean diameter of colonies*	No. of colonies in 1 flask		mean diameter of colonies*
		absolute	%		absolute	%	
1	HeLa K_5	72	36	4,47	144	72	7,29
2	HeLa K_5	47	23,5	4,95	91	45,5	6,01
3	HeLa K_5-3	85	42,6	5,23	68	33,8	7,70
4	HeLa K_5-3	89	46,0	—	81	40,6	—
3	HeLa K_5-8	26	13,0	2,92	29	14,6	4,16
4	HeLa K_5-8	43	21,6	—	56	28,1	—
5	HeLa K_5-8	19	8,5	—	39	19,5	—

*The diameter of the colonies is expressed in units of the ocular micrometer scale (absolute value of one division 0.08 mm).

colonies in accordance with their size in the individual flasks was especially revealing (Fig. 1, A, B, C). It will be apparent from Fig. 1,A that in experiment No. 1 with cells of line K_5 in a medium with 10% serum, the colonies with a diameter greater than 5 scale divisions constituted only 27.9%, and those in a medium containing 25% of serum–79.4% of their total number. Comparison of the cell lines showed that they differed in both criteria. Lines K_5 and K_5-3 differed hardly at all. Clone K_5-8 differed appreciably from them, especially as regards its low seeding efficiency (only half that of K_5-3) and the smaller size of its colonies (two-thirds that of the K_5-3 colonies).

Hence, the higher concentration of serum in the nutrient medium is an important factor favoring the development of a larger number of colonies [8] and promoting a more intensive proliferation of the cells, as a result of which colonies of larger size developed. This last effect of serum was still more constant, thus affording further support for the view that the size of the colonies should be taken into account when assessing the ability of cells to form colonies.

Increasing importance is being attached nowadays to the role of the pH of the nutrient medium in the biology of cell structures [9, 10, 11, 14]. In our experiments the pH of the complete nutrient medium before it was poured into the Carrel flasks was optimal (7.2–7.4). However, after it had been poured into the flasks, its pH shifted towards the alkaline side, sometimes reaching 8.0. When a large number of cells was seeded, this factor was not significant, because the pH rapidly became optimal and then acid as a result of the cell metabolism. In the experiments in which few cells were seeded, a weak alkaline pH adversely affected growth of the colonies. We verified this observation, previously reported in the literature [7, 10], by special experiments using a culture of HeLa K_5 cells. The results are given in Table 2 and in Fig. 1, D and Fig. 2, c, d. In these experiments the development of colonies was compared in a medium with a pH close to 8.0 and in a preliminarily acidified medium, as a result of which the pH lay in the weakly acid zone after the medium had been poured into the flasks (about 7.0). It may be seen from these results that a weakly acid medium was much more favorable for the formation of cell colonies. In this case, as when the serum concentration in the medium was increased, the number of colonies rose and they were larger in size. In a weakly acid medium more single cells apparently start to divide, and they proliferate more energetically [9, 10, 11].

These experiments demonstrated that this method may be used to obtain isolated colonies from single cells. It is most important that the following experimental conditions should be observed: the obtaining of a suspension of single viable cells, the seeding of accurately determined numbers of cells, the use of nutrient media with an increased concentration of serum and with a pH in the weakly acid zone, and analysis of colonies by their number and size after a definite incubation period.

TABLE 2. Development of Colonies of Cells of HeLa Line K_5 in Media of Different pH

Experiment No.	pH 8.0			pH 7.0		
	No. of colonies in 1 flask		mean diameter of colonies*	No. of colonies in 1 flask		mean diameter of colonies*
	absolute	%		absolute	%	
1	98	49	4,80	154	77	6,58
2	91	45	6.08	118	59	5,92
3	59	29	2,87	139	69,5	4,92

*The diameter of the colonies is expressed in units of the ocular micrometer scale (absolute value of one division 0.08 mm).

SUMMARY

An inquiry was made into conditions for the optimal growth of cells of the HeLa strain (parent and four clonic lines) in the form of isolated colonies by cultivating small counted numbers of cells in hermetically sealed Carrel flasks. In particular,a unicellular suspension was used for plating a definite number of cells on nutritive media with increased serum content and with weakly acid pH. Experimental results were evaluated in two weeks not only by the number of developing colonies, but also by their size. The plating efficiency and the size of colonies were much greater in culturing cells on medium No. 199, containing 25% of bovine serum, with the initial pH of about 7.0.

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