CELL GROWTH AS A FUNCTION OF CELL DENSITY

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1. Introduction

It is a common observation that while large numbers of cells from vertebrate animals grow in tissue culture exponentially without delay, small numbers grow poorly if at all. It is the purpose of this paper to set out the numerical and physiological parameters of this restriction on growth initiation, and to relate it briefly to the malignant transformation of normal into cancer cells.

The cells used in these experiments were obtained from 10 day old chicken embryos and ingredients of the medium are:

Nutrient mixture 199	83%
Tryptose phosphate broth	10%
Calf serum	4%
Chicken serum	1%
2.8% $NaHCO_3$	2%.

The experiments on cell growth were done in circular polystyrene plastic petri dishes. The test cells were suspensions of individual cells made by treating three to seven day old primary cultures with trypsin. The cells were counted in a Coulter Electronic Counter.

2. The number of cells required for sustained growth

When the population of cells is about 5×10^4 or greater per 21 cm^2 dish, the cells multiply exponentially without lag with a generation time varying from 16 to 24 hours. At concentrations much below 5×10^4 cells per dish, the growth is much slower, and there may occur a delay of three to four days before any growth is apparent. At concentrations below 10^4 cells per dish, only a small fraction of the cells may sustain their growth sufficiently to form a colony.

The question arises whether the growth of animal cells is dependent on the number of cells per milliliter of nutrient medium or on the density of the cell population on the floor of the dish. It should be noted that normal vertebrate cells in general must attach to a surface and spread thereon before they will grow efficiently. Experiments were carried out using differing volumes of medium, differing surface areas of the culture dish and differing numbers of cells, as follows:

Area	21 cm^2	56 cm^2
Volume	2 ml, 5 ml, 10 ml	10 ml
Initial number of cells	5×10^3, 1, 2, and 5×10^4, 1 and 2×10^5	1, 2, and 5×10^4 1, 2, and 5×10^5.

The number of cells at the end of three, four, and five days were counted.

An analysis of variance was performed to estimate the effects of area, volume, day, and initial number of cells on the logarithm of the number counted, and to test the hypotheses that the particular effects were zero. The hypothesis of no volume effect was not rejected but the other three hypotheses were rejected; the hypothesis of no area effect was rejected at the 0.05 level of significance, no day effect was rejected at the 0.025 level, and no effect of initial number of cells was rejected at a very tiny level of significance. The results of the analysis of

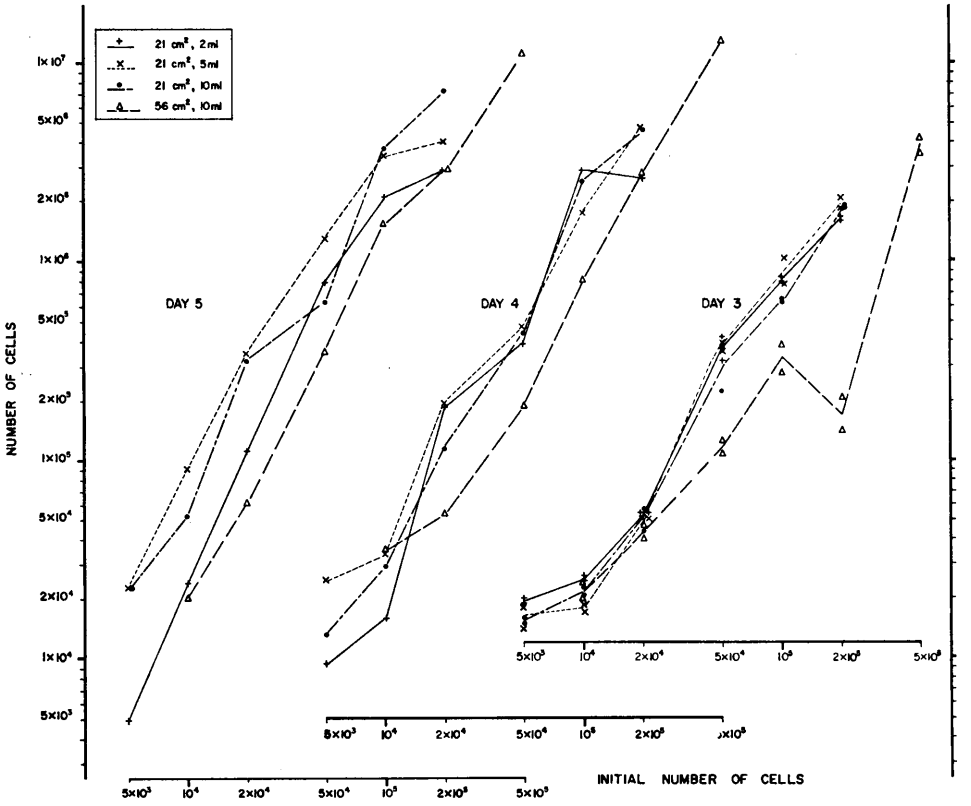

FIGURE 1

Cell growth as a function of area, volume, initial number of cells, and day counted. From right to left are graphs for number of cells on third, fourth, and fifth days after the cells were plated in dishes of area and volume indicated by symbols.

variance show clearly that it is density of the cell population on the floor of the dish (area effect) rather than cells per milliliter of nutrient medium (volume effect) that influences cell growth. The results of this experiment are shown graphically in figure 1.

NOTE ADDED IN PROOF. Much more detailed and extensive experiments have since been conducted by A. Rein and H. Rubin which establish unequivocally that the area effect is the primary determining influence in initiating growth in small cell populations.

The results indicate there is some form of interaction between cells on the surface of the dish. This could be either through the release of a substance which decreases sharply in concentration with distance, or is highly unstable. It will be shown below that a substance which enhances the growth of a small number of cells can indeed be obtained from a large number of cells. The number of cells required to provide optimal concentrations of this substance however is at least ten times higher than the number required to sustain their own growth. The substance is not so unstable that it should not be recovered from the medium at self-sustaining cell concentrations.

The indications are therefore that some intimate form of interaction between cells occurs on the surface of the dish. At first sight this seems incredible because at minimal self-sustaining cell concentrations the area covered by visible portions of the cell amounts to only about 1/50 the area of the entire dish. Under these conditions the average cell is some seven cell diameters distant from its neighboring cells, and it seems ludicrous to envision cell contact as the mediating force. However, this is a case where seeing is not believing. It has been found that a microexudate of protein and lipid containing material extends in a monomolecular film beyond the visible borders of the cell to cover an area 100 times that covered by the cell proper. Thus, invisible extensions of the cell may very well be in contact even when each cell appears to be an isolated island.

NOTE ADDED IN PROOF. Recent experiments by H. Rubin and A. Rein have shown that macromolecules released from cells attached to a solid substratum occur in the fluid medium in a gradient, due primarily to diffusion, which decreases sharply in concentration with increasing distance from the cells.

Of course the distances between cells vary over a wide range and depend on how evenly the cells themselves are distributed over the dish. The variable distribution of intercellular distances is expected to influence the precise nature of the dependence of cellular growth rate on cell density. As a rough guide our results indicate that at cell densities between 10^4 and 10^5 per 21 cm^2 the number of cells in the total population at three and four days is roughly a function of the square of the starting cell population. This suggests that growth is favored by interaction between at least two cells.

3. Physiological basis for cell density effects on growth

The foregoing discussion focuses attention on direct cellular interaction in determining cell growth. There is evidence however that the density requirement

can be overcome by employing a medium which has been "conditioned" in supporting the growth of large numbers of cells. See figure 2. As indicated above,

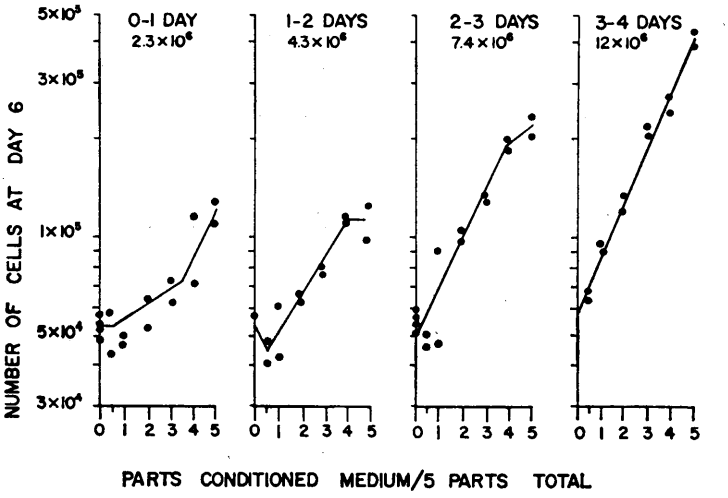

FIGURE 2

Effect of conditioned medium on growth of small number of cells. Fresh medium was conditioned for 24 hours on primary chick embryo cultures during the period indicated in each panel. The conditioned medium was then used to support the growth of 10^4 cells. The number of cells at the end of six days is plotted against the ratio of parts conditioned medium to five parts total. At the top of each panel is the number of conditioning cells.

the size of a cell population required to optimally condition medium to support growth of sparse populations is more than ten times larger than that required for self-sustained growth. The massive excess of cells required to produce effective concentrations of the growth enhancing substance in solution plus the evidence that direct cellular interactions are ordinarily involved in enhanced growth, suggest that the material in solution is a slight "offal" derived from the surface of the cell itself.

The surface of the cell is made up chiefly of lipid and protein. Recent evidence indicates that the lipid and protein moieties of the membrane are combined into structural units as lipoproteins. We may ask therefore whether the growth enhancing material of conditioned medium has the properties of lipoprotein molecules which are known to be large and unstable molecules. The evidence we have obtained so far agrees with the concept that the growth enhancing material is lipoprotein. The biological activity obviously resides in a large molecule or molecular aggregate since it is nondialyzable, can be sedimented in an ultracentrifuge, and is not retained by gel columns which retain molecules of molecular weight less than 30,000 daltons. The biological activity is destroyed by moderate

heating (56°C for 10 minutes), high pH (≧8.5), high salt concentrations (≧0.8 Molar), sonic vibration, and even by stirring. It is also inactivated by very small amounts of surface active agents which are known to disrupt cell membranes.

The biological activity can be said therefore to reside in molecular structures which have all the characteristics of lipoproteins. Efforts are proceeding to purify and rigorously identify the entity responsible for the biological activity. These efforts are complicated by the lability of the biological activity, and by the pre-existing presence of lipoproteins in the original medium. The pre-existing lipoproteins are derived from the serum which is required in the medium. It has become necessary therefore to use radioactive labeling techniques to distinguish pre-existing components of the medium from those synthesized by the cultured cells. These efforts have so far shown us that cells do indeed release lipids and proteins into the medium. Our task now is to ascertain their relationship to the biological activity found in the medium.

4. The conditioning of medium by malignant cells

There are strong indications that some of the most critical aspects of the behavior of malignant cells are the result of changes in the structure of their outer membranes. Malignant cells escape contact inhibition, a process which prevents normal cells in culture from moving over one another. Contact inhibition is mediated by the cell membrane and results from the adhesion of the leading edge of one cell to the edge of another cell. A malignant cell, in not recognizing the constraints of contact inhibition, is free to move over other cells, and this probably accounts for its capacity to invade tissues in the animal, its loss of normal architectural relations to surrounding cells and possibly for its ability to metastasize. Since contact inhibition is determined by the cell membrane it is eminently plausible that the loss of contact inhibition is the result of alterations in the membrane.

While this has been recognized for years, no one has come forward with an effective way for studying the putative membrane alterations. If we assume however that the growth enhancing material in conditioned medium is a component of the normal cell membrane, we might reasonably anticipate that it would be altered in the malignant transformation, and that the alteration would manifest itself in loss of biological activity.

An ideal system for testing the validity of these assumptions is available in the form of the malignant transformation of chick embryo fibroblasts to sarcoma cells following infection by the Rous sarcoma virus (RSV). The unique advantage of this system lies in the capacity of RSV to induce the malignant transformation in a high proportion (80 per cent or more) of cultivated chick embryo fibroblasts within three to four days after infection.

I have studied the growth enhancing activity of medium from cells infected with RSV. The medium was obtained at daily intervals up to one week after

infection. The extent of the malignant transformation was established by close observation of the infected cells in the optical microscope.

The results support our initial expectation. See figure 3. Cells continue to produce the growth enhancing factor at the normal rate for about three days after infection with RSV. The infected cultures usually undergo massive transformation on the third or fourth day and this is accompanied by a disappearance

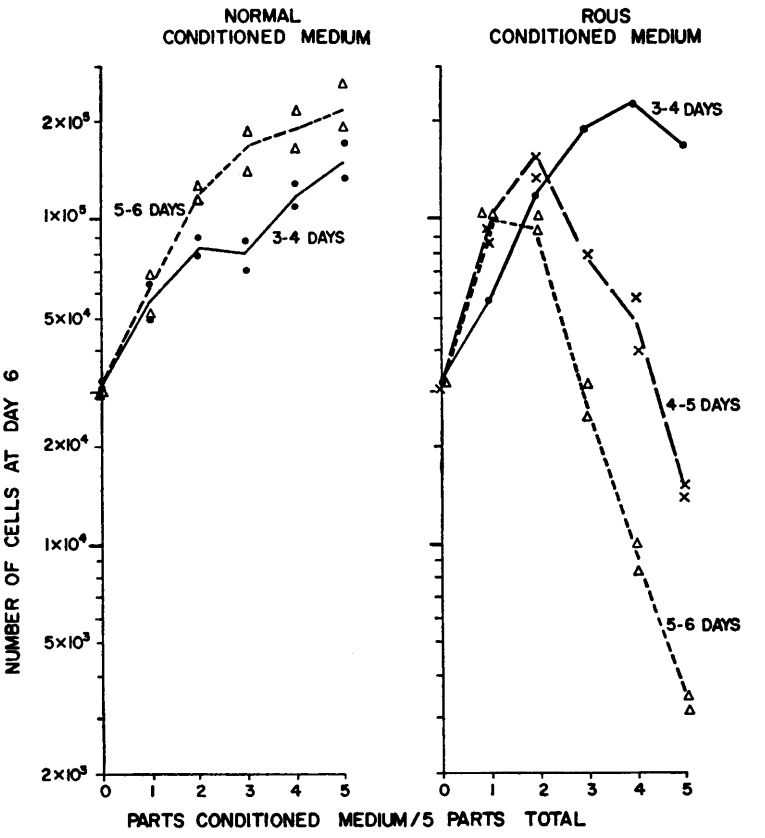

FIGURE 3

Comparison of growth of small number of cells in Rous sarcoma conditioned medium and normal conditioned medium. Each medium was used in various concentrations with fresh medium to support the growth of 10^4 cells. The time interval indicated beside each curve shows when (after cell seeding) the medium was being conditioned.

of growth enhancing activity from the medium. Such media, in fact, became inhibitory to the growth of small numbers of cells. Relatively small quantities of medium obtained from the cells after transformation markedly depress the capacity of a normal conditioned medium to support cell growth.

5. Discussion

The growth of animal cells in tissue culture presents some provocative problems for biomathematical analysis. My intention here is to bring some of these problems to the attention of those more competent than I to carry out the analysis.

The first problem is that of the exact nature of the dependence of cell growth rate on starting cell density. This problem must inevitably deal also with the increased growth rate of some cell clones in small populations as the population grows larger, and the decreased growth rate and death of other cells and cell clones which have accumulated damage associated with a sparse cell population. On the basis of our present knowledge and level of analysis, we can state that the initial growth rate of cells started at densities of 10^4 to 10^5 per culture is proportional to the size of the starting population. More data is needed on the changes in growth rate with time before an intensive analysis is called for. We might reasonably expect to find out the distances over which cells "communicate" and the extent and type of their interaction.

A second problem is that of the dependence of the growth rate of a small number of cells on the concentration of the growth enhancing factor in the medium. So far as we have been able to determine, the growth rate is increased in direct proportion to the concentration of the conditioning factor until the cells achieve a minimum generation time of about 20 hours. The analysis will be complicated by the inhibitory effects of some conditioned media at high concentration.

Are the enhancing and inhibitory effects merely additive? This problem leads directly into the question of the marked inhibition caused by medium from Rous sarcoma cells. Can one determine from a mathematical analysis the number of inhibitory molecules required to affect an enhancing molecule? Does the inhibitory molecule act directly on the enhancing molecule and destroy it, or does it merely have an antagonistic effect on cell growth?

Another analytical problem that arises from this work has to do with the equilibrium postulated to occur between components of the cell membrane *in situ* and free in the medium. This problem must take into consideration the lability of the component when in the medium, and the possibility that aggregation occurs when the component reaches high concentrations.

In any case, I think it is fairly clear that a mathematician would have himself a field day with this system, and I for one would welcome anyone who wants to venture into it.

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[2] ———, "The inhibition of chick embryo cell growth by medium obtained from cultures of Rous sarcoma cells," *Expl. Cell Res.*, Vol. 41 (1966), pp. 149–161.