"CONTACT INHIBITION" OF CELL DIVISION IN 3T3 CELLS*

By Robert W. Holley and Josephine A. Kiernan

BIOCHEMISTRY AND MOLECULAR BIOLOGY, CORNELL UNIVERSITY, ITHACA, NEW YORK

Communicated by Renato Dulbecco, February 26, 1968

The 3T3 cell, an established line of mouse fibroblast cell, has been considered to be extremely sensitive to "contact inhibition" of cell division. Under the usual culture conditions, 3T3 cells grow rapidly in sparse culture, but cell division stops after the cells become confluent, at approximately 10^6 cells per 6-cm dish. The cell monolayer has a typical "cobblestone" appearance. Todaro, Lazar, and Green[1] have described studies of the effect of serum on cell division in these "contact-inhibited" cells. The addition of serum to an inhibited culture leads to a rise in RNA synthesis, followed in a few hours by protein synthesis, and eventually by some DNA synthesis. Todaro, Lazar, and Green[1] have inferred that a factor in serum overcomes "contact inhibition" of cell division. In the experiments described below, we find that the characteristic "contact-inhibited" cell density observed for 3T3 cells is a fortuitous result of growing the cells in a medium that contains 10 per cent calf serum. The final cell density, after cell division stops, is directly proportional to the amount of serum added to the medium.[2] The experiments suggest that serum contributes a factor or factors required by 3T3 cells for cell division. Viral-transformed 3T3 cells have a greatly reduced requirement for the serum factor(s).

Whether serum factors offer an explanation of "contact inhibition" of cell division in other instances remains to be determined. It is pertinent that Temin[3] has found that an insulin-like factor in serum is required for cell divison by cultured chick cells. Transformation of chick cells by Rous sarcoma virus lowers the requirement for this serum factor.

Materials and Methods.—The 3T3 cell line was obtained from Dr. Marguerite Vogt. The cell line had been obtained originally from Dr. Howard Green and had been cloned recently by Dr. Vogt to maintain the typical "cobblestone" appearance. During prolonged culture, 3T3 cells gradually lose their high requirement for serum and grow to higher cell densities. Therefore, the cell line was maintained at $-90°C$, and cells used in the experiments were not cultured over 8 weeks. The cells were grown in enriched Eagle's medium, as used in Dulbecco's laboratory.[4] To count the cells, the medium was removed from the dishes, the cell layer was trypsinized with half the concentration of trypsin used during transfer of the cells, and the cells were counted in the trypsin solution by means of a hemacytometer. Counts were on duplicates. Experiments were replicated at least three times. The standard error observed for replicate counts was approximately 10%.

Assay for growth factor: Approximately 10^5 3T3 cells were plated per 6-cm plastic dish in 5 ml of medium with 6% calf serum. Solutions to be assayed were added 24–48 hr later. (The solutions to be assayed were sterilized by 3-min UV irradiation with a germicidal lamp rather than by filtration, since the growth factor appears to be adsorbed on Millipore filters under some conditions.) Counts of the final number of cells per dish were made at 5 days, usually about a day after growth had stopped.

Partial purification of the growth factor from human urine: The urine was frozen immediately, to avoid microbial action, and was lyophilized. The residue was dissolved in water to give one tenth the original volume, and the solution, with suspended solids, was dialyzed overnight at 4°C against 0.05 *N* sodium chloride, in the presence of chloroform.

The dialyzed solution was centrifuged to remove insoluble material and was lyophilized. The residue from the second lyophilization was redissolved in water to give 1% of the original volume of urine. Solids were removed by centrifugation, and approximately 4.5 ml of the clear solution was chromatographed on a 2 × 30-cm column of Sephadex G 25 (Fine) packed in 0.1 *N* NaCl. The active material was excluded by the gel and came off the column in association with the initial ultraviolet-absorbing light yellow band. Fractions comprising the initial peak (total of 15–20 ml) were combined, and the active material was adsorbed on a 0.7 × 25-cm column of DEAE-cellulose (Whatman, microgranular, DE32) packed in 0.1 *M* Tris–chloride buffer, pH 7.5. The column was eluted with a linear gradient prepared from 40 ml of 0.1 *M* Tris–chloride, pH 7.5, and 38 ml of 1 *N* NaCl in the Tris buffer. The activity for growth of 3T3 cells came off the column just after the main ultraviolet-absorbing band, at approximately 0.5 *N* NaCl. Solutions of the growth factor were stored at −20°C.

Results and Discussion.—Preliminary experiments, in which serum was added after the 3T3 cells had become confluent, were consistent with the results described by Todaro, Lazar, and Green.[1] However, interpretation of the results was changed by experiments in which extra serum was added early in the growth of the cultures. Figure 1 shows growth curves of 3T3 cells in media that contained 10, 20, and 30 per cent calf serum. The final cell count varies directly with the amount of serum added.[2] The growth curves in the presence of high serum show no evidence of a break in growth rate when the cells become confluent at approximately 6 × 10^5 cells per dish.

If 3T3 cells are grown in medium that contains 30 per cent calf serum and the medium is changed frequently, the cells grow to a density of approximately 6 × 10^6 cells per dish before the cell layer detaches. At this high cell density, which is approximately ten times the density of a confluent monolayer, the 3T3 cells are very tightly packed and are piled on top of each other.

It is clear, therefore, that 3T3 cells grow readily to cell densities far above the density of a confluent monolayer. Nevertheless, different interpretations of the

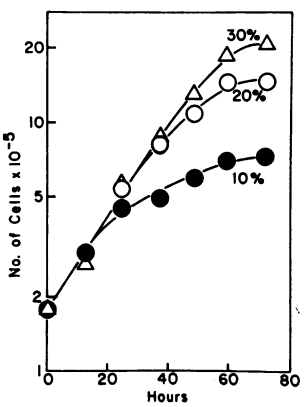

FIG. 1.—Growth curves of 3T3 cells in media containing 10, 20, and 30% calf serum.

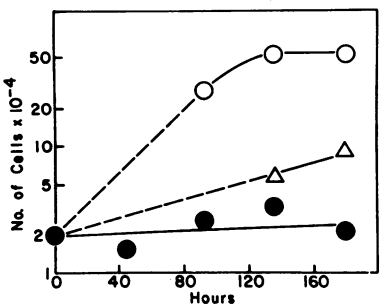

FIG. 2.—Growth curves of 3T3 cells in medium depleted by growth of 3T3 cells until growth stopped: *solid circles*, without fresh calf serum; *open circles*, with 10% fresh calf serum; *triangles*, depleted medium replaced at 20 hr and at 90 hr with medium depleted by a 3-day exposure to confluent 3T3 cells.

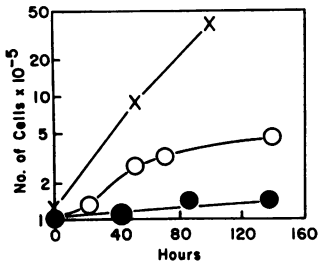

FIG. 3.—Growth curves in medium depleted by growth of 3T3 cells until growth stopped; *solid circles*, 3T3 cells without fresh calf serum; *open circles*, 3T3 cells with 10% fresh calf serum; *crosses*, SV3T3 cells without fresh calf serum.

results are possible. One interpretation is that serum contains a growth factor that is required by 3T3 cells. Alternatively, serum may contain a factor that overcomes contact inhibition. To distinguish between these two alternatives, studies were made of the growth of sparse 3T3 cells in depleted medium. As shown in Figures 2 and 3, sparse 3T3 cells, plated at approximately 10^4 or 10^5 cells per dish, grow very slowly in medium taken from confluent 3T3 cells, unless fresh calf serum is added. In contrast to the behavior of 3T3 cells, SV 40-viral-transformed 3T3 cells (SV3T3 cells) grow very well in the depleted medium (Fig. 3).

In most of these experiments, sparse cells were plated in medium that had been depleted by growth of 3T3 cells until growth had stopped. Growth of sparse cells is significantly better in medium that has been depleted by a three-day exposure to confluent cells (Fig. 2), though growth is still poor compared with that observed after the addition of serum. It is possible that the method of depletion of the medium is responsible for the variation between our results and those of Todaro, Lazar, and Green[1] that sparse cells grow to confluency in depleted medium.

If serum contains a growth factor that is required by 3T3 cells, it should be possible to limit growth at a cell density below the confluent cell density simply by limiting the amount of serum in the medium. This can be done easily. In medium that contains 1 per cent serum, growth of 3T3 cells stops at approximately 10^5 cells per dish.

The growth of 3T3 cells under the usual culture conditions thus seems to be limited by the exhaustion of one or more factors in serum. The active material in calf serum is nondialyzable, and appears to be of relatively high molecular weight (approximately 100,000) by gel filtration. Activity is lost on pronase treatment of the serum. Serum heated in a boiling water bath forms a semisolid product that is inactive in supporting the growth of 3T3 cells, but there is a possibility that active material is trapped within the gel.

Mouse serum has been found to be approximately ten times as active as calf serum, and rat serum has intermediate activity, suggesting that the active material may show some species specificity. Serum from adrenalectomized rats and from thyroidectomized rats has normal activity. Serum from hypophysectomized rats has approximately half the activity of normal rat serum one week after hypophysectomy. Assays of a number of commercial preparations of hormones, including ACTH, follicle-stimulating hormone, gonadotropin, growth hormone, insulin, luteinizing hormone, oxytocin, and prolactin, have been negative.

Commercial thyrotropic hormone (Sigma Chemical Co.) has been found to contain considerable activity. It is approximately 100 times as active as calf

serum, on a milligram protein basis; however, the fact that the amount of the preparation required to give a significant growth response (approximately 0.1 unit of thyrotropic hormone activity) is at least 100 times the amount of thyrotropic hormone expected[5] in the volume of serum that has equivalent activity suggests that the active material is an impurity in the thyrotropic hormone preparation. The active material in the commercial thyrotropic hormone preparation has the same gel-filtration properties as the active material in calf serum. The activity is destroyed by pronase. In contrast with serum, a solution of the thyrotropic hormone preparation can be heated in a boiling water bath ten minutes without coagulation, and most of the activity for growth of 3T3 cells survives.

Commercial human chorionic gonadotropin (Sigma Chemical Co.) also contains activity. Again, the amount of activity is low enough to suggest that the active material is an impurity in the preparation.

The fact that human chorionic gonadotropin is isolated from pregnant urine suggested that the active material might be a normal constituent of urine. Urine turns out to be an excellent source. The activity of rat urine and human urine, on a volume basis, is approximately equal to that of serum, though urine is toxic at high levels. The active material in urine has been concentrated and partially purified by lyophilization, dialysis, lyophilization, gel filtration, and DEAE-cellulose chromatography as described in the *Materials and Methods* section. Table 1 summarizes the results. With 3T3 cells, the isolated material gives a growth response at the microgram level. The activity is destroyed by pronase, but is relatively stable to heating at 100°. The active material isolated from urine is excluded by Sephadex G25, but has a significantly lower molecular weight than the active material in serum.

The maximum growth of 3T3 cells obtained by the addition of the growth factor isolated from urine or from commercial thyroptropic hormone is less than the maximum growth that can be obtained by the addition of calf serum. This suggests that there are other factors in calf serum that are required by 3T3 cells. This possibility is being investigated.

Summary.—Evidence is presented that under the usual conditions for culture of 3T3 cells, exhaustion of essential growth factor(s) present in the serum in the medium is responsible for cessation of growth (contact inhibition of cell divi-

TABLE 1. *Purification of growth factor from urine.*

Additions to 5 ml medium	A_{280} units*	No. of cells per 6-cm dish ($\times 10^{-5}$)	Relative activity†
None (control)	—	6.6	—
0.5 ml calf serum	38	16.4	1
0.2 ml human urine	15	11.5	1.3
0.02 ml after second lyophilization	2.3	11.8	9
0.01 ml Sephadex-G25 column fraction	0.02	9.0	450
0.02 ml DEAE-cellulose column fraction	0.004	9.6	2900

* One unit of absorbance at 280 mμ is defined as the amount of material that gives an absorbance of 1.0 at 280 mμ when dissolved in 1.0 ml and with a light path of 1.0 cm.

† The increase in number of cells (above the control) per A_{280} unit of material, with the increase observed for calf serum set equal to one.

sion) as the cells become crowded. Viral-transformed 3T3 cells have a greatly reduced requirement for the serum factor(s) and thus do not show "contact inhibition" of cell division. Material that is active in stimulating the growth of 3T3 cells has been found in commercial preparations of thyrotropic hormone and human chorionic gonadotropin, and in urine. A procedure for partial purification of the active material from human urine gives material that shows growth-stimulating activity at the microgram level.

* The work was started in Dr. Renato Dulbecco's laboratories at the Salk Institute for Biological Studies. The advice of Dr. Dulbecco and Dr. Marguerite Vogt is gratefully acknowledged. The work was supported by the National Science Foundation.

[1] Todaro, G. J., G. K. Lazar, and H. Green, *J. Cell. Comp. Physiol.*, **66**, 325 (1965).

[2] These results are in agreement with a recent report by Todaro, G., Y. Matsuya, S. Bloom, A. Robbins, and H. Green, in *Growth Regulating Substances for Animal Cells in Culture*, ed. V. Defendi and M. Stoker (Philadelphia: Wistar Institute Press, 1967), p. 87.

[3] Temin, H. M., *J. Cell Physiol.*, **69**, 377 (1967); in *Growth Regulating Substances for Animal Cells in Culture*, ed. V. Defendi and M. Stoker (Philadelphia: Wistar Institute Press, 1967), p. 103.

[4] Vogt, M., and R. Dulbecco, these PROCEEDINGS, **49**, 171 (1963).

[5] Robbins, J., J. E. Rall, and P. G. Condliffe, in *Hormones in Blood*, ed. C. H. Grace and A. L. Bacharach (New York: Academic Press, 1961).