GROWTH CONTROL IN CULTURED 3T3 FIBROBLASTS

Assays of Cell Proliferation and Demonstration of a Growth Inhibitory Activity

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ABSTRACT

Treatment of sparse, proliferating cultures of 3T3 cells (target cells) with medium conditioned by exposure to density-inhibited 3T3 cultures resulted in an inhibition of growth and division in the target cells when compared to similar treatment with unconditioned medium (UCM). This differential effect of conditioned medium (CM) and UCM on target cells was demonstrated using three assay systems: (*a*) assessment of total cell number; (*b*) measurement of [^{3}H]thymidine incorporated into acid-precipitable DNA; and (*c*) determination of the percentage of radioactively labeled nuclei in individual cells after incorporation of [^{3}H]thymidine. The difference in the total incorporation of [^{3}H]thymidine in CM-treated and UCM-treated cells was reflected by a difference in the percent of labeled cells. There was no difference in the average number of grains per labeled cell in the two cultures. Moreover, the inhibitory effect of the CM on target cell proliferation was reversible. Finally, this growth inhibitory activity can be collected in serum-free medium, precipitated by ammonium sulfate, and fractionated by gel filtration. In these purification procedures, the inhibitory activity was consistently found to be associated with the protein-containing fractions of the CM. No activity was found upon similar treatment with UCM. These results suggest that a system has been developed for the purification and molecular analysis of growth inhibitory factors that may mediate growth control in culture fibroblasts.

KEY WORDS density-dependent growth inhibition · cell cycle controls · DNA synthesis regulation · soluble growth regulators · conditioned medium

Most normal cells have two alternative modes of existence, quiescent and proliferative. The growth and division of these normal cells is usually well regulated by the action of various endogenous stimulators and inhibitors. In contrast, most cells from neoplastic sources are relatively insensitive to the regulatory program and are unable to shift into the quiescent state (19, 20, 22). A number of in vitro systems amenable to the study of activation of resting cells to undergo DNA synthesis and cell division have been developed and many growth-promoting substances have been isolated and characterized (8). Much less progress has been made, however, in establishing a well-defined system for the study of endogenous growth inhibitors that may function in cellular homeostasis (12). Although growth inhibitory activity has been demonstrated in crude extracts from a number of tissues, much remains to be developed so that

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purification and characterization of these factors can be accomplished.

In our studies directed at some of these problems, we have chosen to analyze the growth properties of 3T3 fibroblasts (30), a cell line that is highly responsive to density-dependent inhibition of growth (5, 7, 31). The basis of our studies is derived from three key observations: (*a*) In attempting to establish keratinizing epidermal cells in culture from human skin explants or a mouse teratoma cell line, Rheinwald and Green (23, 24) found that skin or teratomal fibroblasts consistently overgrew the epidermal cells. The growth of these fibroblasts was much suppressed, however, by the presence of a feeder layer of lethally irradiated 3T3 cells. In contrast, the growth of the epithelial cells was not affected by the 3T3 cells. (*b*) When a cover slip containing growing 3T3 cells was co-cultured with a cover slip containing density-inhibited 3T3 cells, the growth and phosphate metabolism of the cells on the sparse cover slip was suppressed (9). (*c*) Yeh and Fisher (35) have demonstrated that, whereas the addition of fresh growth medium to contact-inhibited cells stimulated a wave of DNA synthesis and cell division, medium conditioned by exposure to confluent cultures of 3T3 cells could no longer induce the growth of the same target cells. These results are consistent with the notions that either the depletion of medium components or the release of a soluble inhibitor may be responsible for mediating density-dependent inhibition of growth.

In this paper, we demonstrate that treatment of sparse, proliferating cultures of 3T3 cells (target cells) with medium conditioned by exposure to density-inhibited 3T3 cultures (source cells) resulted in an inhibition of growth and division in the target cells when compared to similar treatment with unconditioned medium (UCM).[1] We have characterized some properties of the 3T3 system for the preparation and assay of the growth inhibitory activity. In addition, we have performed fractionation studies that have resulted in the enrichment of the growth inhibitory factor. The results suggest that a well-defined system for the molecular characterization of these factors and for the analysis of their mechanisms of action in growth regulation may be developed from the 3T3 cell line.

[1] *Abbreviations used in this paper:* CM, conditioned medium; CM (SF), conditioned medium prepared in the absence of fetal bovine serum; DME, Dulbecco Modified Eagle's Medium containing 100 U/ml of penicillin and 100 μg/ml of streptomycin; DME-FBS, DME supplemented with fetal bovine serum to a final concentration of 10%; PBS, phosphate-buffered saline (8.00 g NaCl, 0.20 g KCl, 1.15 g Na_2HPO_4, 0.20 g KH_2PO_4 per liter, pH 7.4); UCM, unconditioned medium; UCM (SF), unconditioned medium prepared in the absence of fetal bovine serum.

MATERIALS AND METHODS

Cell Culture

Experiments were performed with Swiss 3T3 fibroblasts obtained from American Type Culture Collection (Rockville, Md.) or from Dr. Howard Green (Massachusetts Institute of Technology, Cambridge, Mass.) with comparable results. The cells were cultured in Dulbecco Modified Eagle's Medium (K. C. Biological, Lenexa, Kans.) containing 10% fetal bovine serum (Grand Island Biological Co. [GIBCO], Grand Island, N. Y.) and 100 U/ml penicillin and 100 μg/ml streptomycin (DME-FBS). They were incubated in Corning plastic tissue culture flasks (Corning Glass Works, Science Products Div., Corning, N. Y.) maintained at 37°C in a humidified atmosphere of 10% CO_2. These fibroblasts were passaged and used for a maximum of 3 mo, at which time a fresh sample was initiated from cultures kept frozen at −80°C.

Under our culture conditions, the doubling time and saturation density for the 3T3 cells were 22 h and 4–5 × 10^4 cells/cm^2, respectively (see reference 21). The cells were passaged by dislodging them from the growth surface using a 0.25% trypsin (Nutritional Biochemicals, Cleveland, Ohio) solution in phosphate-buffered saline (PBS) containing 4 × 10^{-4} M versene. After centrifugation the cells were resuspended in DME-FBS and seeded at a density of 4 × 10^3 cells/cm^2. The 3T3 cells were monitored for mycoplasma contamination by autoradiography and by the agar culture technique (16). Both assays indicated that our 3T3 cells were free of mycoplasma.

The other cells used in our studies were: SV40 virus-transformed 3T3 cells (31), hamster fibroblast cell lines Nil-2 and polyoma virus-transformed Nil-2 cells (4), neuroblastoma cell line N4TG3 (2), rabbit kidney cells, LCC-RK_1 (14), and secondary chicken embryo fibroblasts obtained from 10-d-old eggs following the procedure described (15). All cells were cultured in DME-FBS.

Preparation of Conditioned Medium

Conditioned medium (CM) was prepared according to the protocol diagrammed in Fig. 1. Source cells were grown to a confluent monolayer. Fresh DME-FBS was then added ("stimulation feeding") to insure complete confluence of the culture. After 24 h, the medium was again removed, fresh DME-FBS was added, and the flasks were incubated at 37°C for 24 h. This medium was centrifuged at 1470 *g* for 10 min and the supernatant was used as CM. In all experiments, UCM was prepared in parallel from the same batch of DME-FBS and was incubated under the same conditions but in the absence of any cells. Serum-free conditioned medium [CM (SF)] and serum-free unconditioned medium [UCM (SF)] were prepared following the same protocol except that the DME was not supplemented with fetal bovine serum during the period of conditioning. These were supplemented with serum to a final concentration of 10% just before they were tested on target cells.

Target cells were routinely seeded at an initial density of 2.5

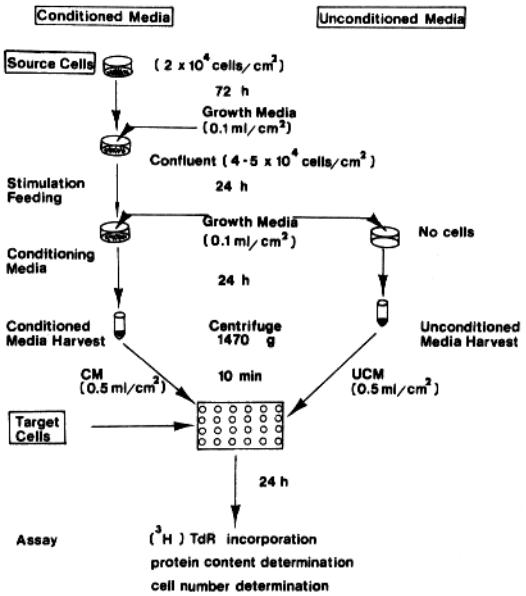

FIGURE 1 Schematic diagram of the protocol used in the preparation of CM and UCM. The cell densities are expressed as the number of cells per square centimeter of growth surface in the tissue culture dish. Similarly, the volume of medium added at the various steps are expressed in this diagram as the number of milliliters of medium used per unit area of growth surface. The details of this protocol are described in the text under Materials and Methods.

$\times 10^3$ cells/cm^2 in 24-well tissue culture dishes (2 cm^2/well, Costar, Cambridge, Mass.). After 24 h, the growth medium was removed, and 1 ml of CM or UCM was added. The growth of these target cells treated with CM or UCM was determined using the assays to be described below. Target cells were always taken from our stock cultures that have never reached high densities. Source cells were discarded after they have been used twice for the preparation of CM.

Assays of DNA Synthesis

Cells were pulsed with 1 μCi/culture of [^{3}H]thymidine (1.9 Ci/mmol, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) for 3 h at 37°C. After the pulse, the cells were washed three times with cold PBS, removed from the culture dish by trypsin treatment, and deposited on Whatman GF/A filters. The filters were washed with PBS, 5% trichloroacetic acid, and methanol before they were immersed in 5 ml of cocktail (1 g dimethyl-1,4-bis[2-5-phenyloxazolyl]benzene; 8 g 2,5-diphenyloxazole; 1,333 ml Triton X-100; and 2,666 ml of toluene) for scintillation counting.

For autoradiography, cells were cultured in Leighton tubes (5 cm^2/slide, Costar) at an initial density of 4×10^3 cells/cm^2. After 1 d, the cells were treated with UCM and CM for 24 h and then pulsed with 0.1 μCi/culture for 3 h at 37°C. The cells were washed five times with cold PBS, 5% trichloroacetic acid, and fixed in abolute ethanol. The Leighton tube slides were then dipped in NTB-2 Nuclear Track emulsion (Eastman Kodak Co., Rochester, N. Y.) and exposed for 18–24 h (25). After final fixation, slides were stained in Giemsa (GIBCO), dipped in xylene, and a cover slip was mounted with Permount (Fisher Scientific Co., Pittsburgh, Pa.).

Slides were scanned systematically and at least 70 labeled cells were counted in every case. Multiple counts of the grains in the same cell indicated that the standard error of the visual grain counting method is 5–10% in each individual measurement. For the reversibility experiments, the amount of [^{3}H]thymidine in each culture was increased to 0.2 μCi and the labeled cells were exposed to the photographic emulsion for 48 h. These procedural modifications facilitated the counting of percent labeled cells.

Assays of Cell Viability and Cell Number

The viability of cells treated with UCM and CM were determined while the cells remained attached to the plastic growth surface. After removal of growth medium, the cells were incubated with trypan blue (0.08% in PBS) for 10 min at room temperature. The staining solution was then removed and the viable cells were counted using an Olympus inverted microscope (Olympus Corp. of America, New Hyde Park, N. Y.).

Target cells were also labeled with ^{14}C-amino acids (0.75 μCi/culture, 58 Ci/matoms of ^{14}C, Amersham Corp., Arlington Heights, Ill.) for 48 h. After washing, these cells were treated with UCM or CM. At various times thereafter, the cells were washed with PBS and were dissolved in 100 μl of 0.1% sodium dodecyl sulfate. The amount of labeled target cells remaining after UCM and CM treatment was determined by counting the ^{14}C radioactivity in each culture.

Two different methods were used to determine the total number of cells in a given culture. In the first method, the cells were washed three times with PBS and were removed from the growth surface by treatment with trypsin (0.25%, 5 min, at 37°C). After centrifugation, the cells were resuspended in PBS containing trypan blue and counted in a corpuscle counting chamber (Hausser Scientific, Blue Bell, Pa.). Alternatively, the cells in culture dishes were washed with PBS and then dissolved in 0.1% sodium dodecyl sulfate. An aliquot of this sample was then used for determination of total protein in the culture following the procedure described by Lowry et al. (17). Bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.1% sodium dodecyl sulfate was used for calibrating the standard curve. In both UCM and CM cultures, the amount of protein was directly proportional to the number of cells.

Co-cultivation of Dense and Sparse Cultures

In these experiments, cells were seeded on glass cover slips (2.5 × 1 cm) cut from microscope slides. For cover slips containing dense cultures ($\sim 5 \times 10^4$ cells/cm^2), 3T3 cells were inoculated at 2×10^4 cells/cm^2 and allowed to grow for 72 h. For cover slips containing sparse cultures ($\leq 10^4$ cells/cm^2), the cells were seeded at 2.5×10^3 cells/cm^2 and cultured for 24 h. Both types of cover slips were then incubated with 0.75 μCi/culture of ^{14}C-labeled amino acids for 24 h. After washing three times with DME-FBS, the cover slips were then moved into a new culture dish (10 cm^2/well, Costar) in various combinations and 1.5 ml of fresh DME-FBS was added to each dish. The cultures were shaken on a turntable at 30 rpm. After 24 h of co-culture, DNA synthesis was assayed as previously described except the cells on each individual cover slip were scraped into 2 ml of PBS and then deposited on Whatman GF/A filters.

Fractionation of CM Components

For the fractionation studies, solid ammonium sulfate was

added to 40 ml of CM (SF) to 70% of saturation at room temperature. All subsequent operations were performed at 4°C. The supernatant and precipitate fractions were separated after centrifugation for 15 min at 12,400 g. The precipitate was resuspended in 3 ml of DME. Both the supernatant and precipitate fractions were dialyzed against PBS (4 liters, 3 h), PBS (2 liters, 1.5 h), and DME (1.5 liters, 1.5 h). Cellulose dialysis tubing (Arthur H. Thomas, Philadelphia, Pa.) with a mol wt cutoff of 12,000 was used. Similar procedures were carried out on UCM (SF); in this case, no precipitate was observed after ammonium sulfate addition and centrifugation. Fetal bovine serum was added to all fractions before they were tested on target cells.

For column fractionation, the ammonium sulfate precipitate was redissolved in 1 ml of DME and applied to a column (1 × 60 cm) of Sephadex G-15 equilibrated with DME. Fractions of 1 ml were collected and assayed for protein content (50-μl aliquots of each fraction) using the Lowry method (17) and bovine serum albumin in DME as standards. The remainder of each fraction was combined with 0.1 ml of fetal bovine serum and tested on target cells. In both the CM (SF) and UCM (SF) fractionations, the ammonium sulfate appeared as a wide zone at an elution volume corresponding to the total volume of the column. The application of these fractions to target cells was found to lyse the cells. Therefore, the presence or absence of growth inhibitory activity in these fractions could not be ascertained.

RESULTS

Inhibition of DNA Synthesis in Sparse 3T3 Cells Co-cultured with Dense Cells

The proliferative properties of sparse 3T3 cells in the presence of a density-inhibited 3T3 culture were studied using the experimental scheme of Harel et al. (9). Two cover slips inoculated at different cell densities were placed in the same petri dish containing fresh DME-FBS (Table I). DNA synthesis in cells on a cover slip containing a sparse culture was greatly suppressed when the cover slip was co-cultivated with another cover slip containing a dense culture. This inhibition was not observed when two cover slips both containing sparse cultures were incubated together. These data on DNA synthesis are in agreement with the results of Harel et al. (9) who measured cell growth and phosphate metabolism using the same experimental scheme. Together, the results indicate that density-inhibited 3T3 cells could strongly influence the growth of neighboring sparse cultures, either by depleting medium components or by passing out soluble inhibitory factors.

Effect of CM on the Proliferative Properties of Target Cultures

To analyze this problem further, sparse cultures of 3T3 cells (target cells at 5×10^3 cells/cm^2) were treated with CM (Fig. 1). DNA synthesis in these CM-treated cells was markedly inhibited when compared to parallel cultures treated with UCM (Fig. 2). The [^{3}H]thymidine incorporation was also assayed at the level of individual cells by autora-

TABLE I

DNA Synthesis in Sparse Cultures of 3T3 Cells Co-cultivated with Density-inhibited Cells

Exp*	Cell density of cover slip	DNA synthesis‡	Protein content§	DNA synthesis/protein content
		cpm	*cpm*	
SD	S	1,428	548	2.6
	D	5,357	1,930	2.8
SS	S	6,493	368	17.6
	S	7,556	477	15.8
DD	D	7,527	2,288	3.3
	D	7,753	3,453	2.2
S	S	8,258	606	13.7
D	D	7,172	3,104	2.3

* The abbreviations used for the different types of cultures are: S, sparse cultures (≤10^4 cells/cm^2); D, dense cultures (≥5×10^4 cells/cm^2).

‡ DNA synthesis was measured by the amount of [^{3}H]thymidine incorporated into acid-precipitable material; the reported values represent the averages of results from triplicate cultures.

§ The protein content of the individual cover slips during the pulse period of [^{3}H]thymidine incorporation was determined by the amount of radioactivity that was due to ^{14}C-labeled amino acids incorporated before the co-cultivation experiment. The reported values represent the averages of results from triplicate cultures.

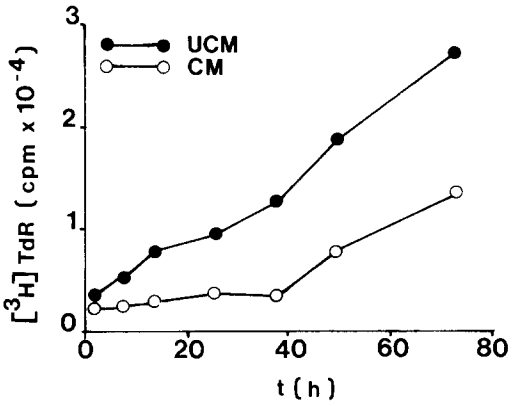

FIGURE 2 The kinetics of [^{3}H]thymidine (*[^{3}H]TdR*) incorporation in 3T3 cells cultured in the presence of CM (○) and UCM (●). At various times, parallel cultures were pulsed with 1 μCi of [^{3}H]thymidine for 3 h. Data points are the averages of measurements on duplicate cultures and are plotted at times corresponding to 1.5 h after the start of each pulse.

diography. In UCM-treated cultures, ~56% of the nuclei were labeled, as compared to only 35% in CM-treated cultures. The fraction of labeled nuclei decreased to 29% when cultures were treated with CM for 48 h.

More importantly, the average number of grains per labeled cell was invariant within the error of estimation for CM- and UCM-treated cultures. The distributions of grain counts for labeled cells of both cultures appeared to be similar (Fig. 3). Chi-square analysis of the data in Fig. 3 showed that these distributions are the same at the 90% confidence level. Therefore, the inhibition of [^{3}H]thymidine incorporation in target cells treated with CM reflects a reduction of the percent of cells undergoing DNA synthesis rather than alterations of the transport or pool sizes of the label.

These results were confirmed by comparing the number of cells in target cultures treated with UCM and CM (Fig. 4). Target cells exposed to UCM continued to proliferate up to the characteristic saturation density (5×10^4 cells/cm^2). In contrast, cultures exposed to CM showed much smaller increases in their cell numbers.

We have also observed similar growth inhibitory activity in CM prepared in the absence of any serum [CM (SF)]. Furthermore, ammonium sulfate precipitation and gel filtration studies indicate that the observed inhibition is not due entirely to the depletion of medium components. We shall describe these experiments in the last part of Results.

Viability and Reversibility of the Cells Treated with CM

Control experiments were performed to ascertain that the difference in the response of target

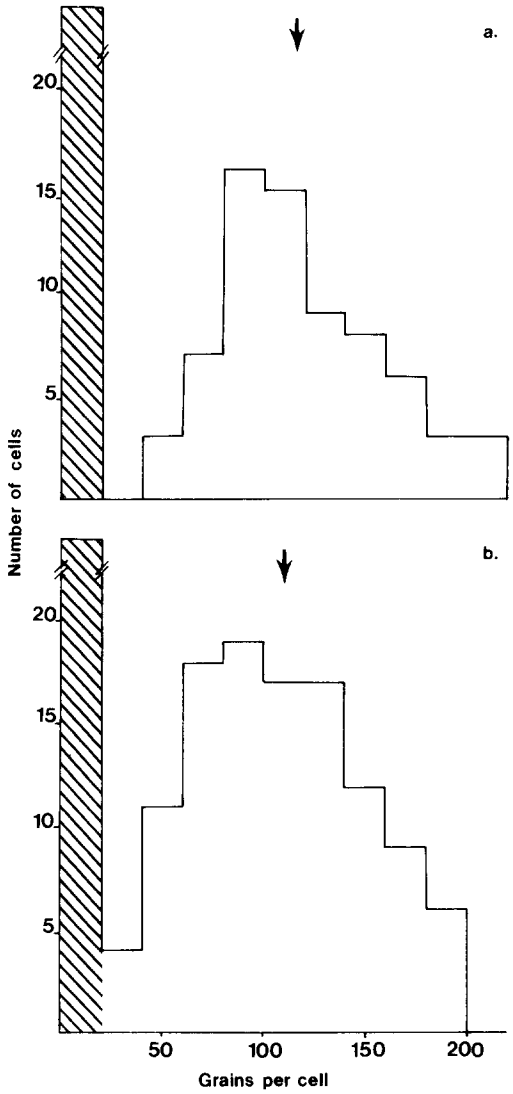

FIGURE 3 Grain count distributions for 3T3 cells cultured in the presence of (*a*) CM and (*b*) UCM. The following numerical data were obtained for CM cultures in *a*: 200 total cells counted, 70 labeled cells (35% labeled cells), average number of grains per labeled cell, 115. The corresponding data for UCM cultures in *b* were: 200 total cells counted, 111 labeled cells (56% labeled cells), average number of grains per labeled cell, 108. The average number of grains per labeled cell for each of the cultures is indicated by the arrow in the grain count distribution graphs in *a* and *b*.

cells to UCM and CM was due to a true suppression of cell growth rather than to any cytotoxic effects of CM (Table II). First, the viabilities of cells, assayed by trypan blue exclusion tests, were identical for target cultures treated with UCM and CM for up to 48 h. Because nonviable cells could have been lost from the dishes, we prelabeled the target cells by culturing in the presence of ^{14}C-amino acids. After treatment with UCM or CM, the levels of ^{14}C label retained were found to be similar in both sets of cultures (Table II). Finally, there was no significant difference in the morphology of the target cells treated with UCM or CM.

To demonstrate that the inhibitory effect of CM on DNA synthesis was reversible, parallel target cultures were treated with CM for 24 h. The medium was then removed and replaced with an equal volume of fresh DME-FBS. At various times thereafter, DNA synthesis was assayed by the incorporation of [^{3}H]thymidine. Concurrent experiments were also carried out with UCM treatment. The data showed that the inhibition was reversible within 10 h after removal of CM (Fig. 5*a*). Similar results were obtained when target cells were exposed to CM for 12, 48, and 72 h before replacement with fresh DME-FBS.

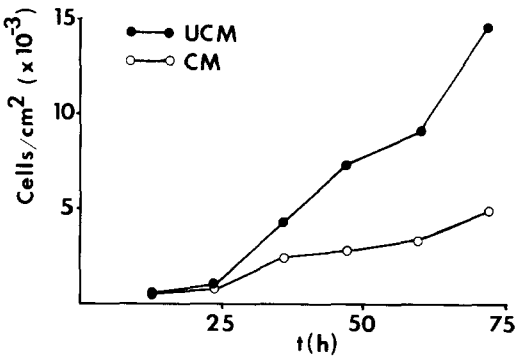

FIGURE 4 The kinetics of the increase in cell density of 3T3 cells cultured in the presence of CM (○) and UCM (●). At various times, parallel cultures were trypsinized and the cells collected for determination of the total cell number in the cultures. Data points are the averages of measurements on duplicate cultures.

The reversibility of the CM effect has also been demonstrated by autoradiography. The percentage of radioactively labeled cells increased from 32% in cells treated with CM for 24 h to ~55% in cells replenished with fresh DME-FBS (Fig. 5*b*). In contrast, control cultures treated with UCM showed labeling in ~45% of the cells throughout this experiment. Taken together with the previous demonstration that the viability of target cells was not affected by CM, these results suggest that the inhibitory activity of CM cannot be ascribed to cytotoxicity.

Source Cell Parameters

To determine the optimal conditions for the preparation of CM, the effects of varying the following parameters were studied: (*a*) variation of the density of source cells used to condition a constant volume of medium (Fig. 6*a*); (*b*) variation in the volume of medium conditioned by a constant number of source cells at the saturation density (Fig. 6*b*); (*c*) variation of the volume of the medium and the source cell density proportionally (Table III); and (*d*) variation in the length

TABLE II

Effect of CM and UCM on the Viability of Target Cells

	Cell viability*		Retention of prelabeled ^{14}C proteins‡	
Time	UCM	CM	UCM	CM
h	*No. stained cells/total cells counted*		*cpm*	
0	0/212	0/100	4,238 ± 226	4,201 ± 114
3	0/202	1/233	4,274 ± 187	3,929 ± 143
6	0/229	0/282	3,321 ± 130	3,325 ± 171
24	1/228	1/285	2,686 ± 91	2,601 ± 271
48	0/202	0/216	2,028 ± 19	1,984 ± 75

* Cell viability was assayed by the trypan blue dye exclusion technique described in Materials and Methods.

‡ Target cells were cultured in ^{14}C-labeled amino acids for 48 h, washed, and then treated with UCM and CM. At the times indicated, the amount of labeled target cells was determined by counting the ^{14}C radioactivity in each culture. The data are expressed as averages of triplicate determinations ± SEM.

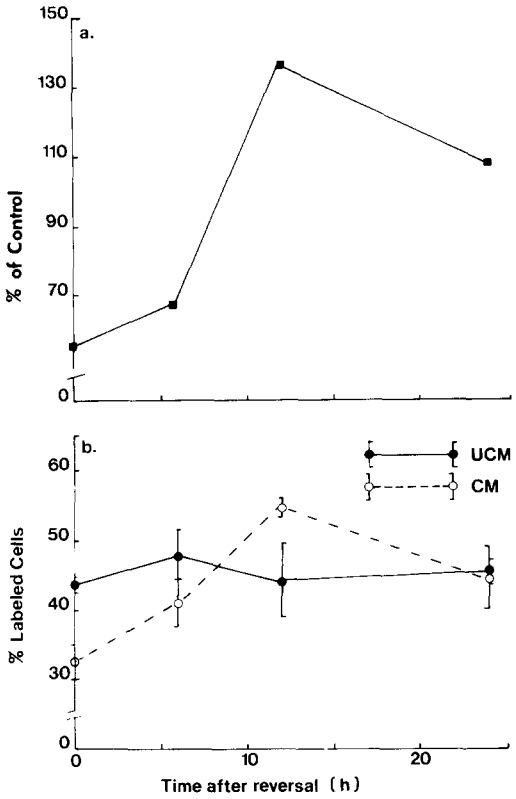

FIGURE 5 The reversibility of the effect of CM. Target cells (5×10^3 cells/cm^2) were treated for 24 h with CM and UCM. The medium was then removed from each culture and replaced with an equal volume of fresh DME-FBS. (*a*) At various times thereafter, DNA synthesis was assayed by the incorporation of [^{3}H]thymidine, normalized by the protein content of the culture, and expressed as percent of control ([^{3}H]thymidine incorporation per milligram protein in CM cultures divided by [^{3}H]thymidine incorporation per milligram protein in UCM cultures). Data points represent the averages of measurements on duplicate cultures. (*b*) Percent of radioactively labeled cells as determined by autoradiography in cultures treated with CM (○) and UCM (●) at various times after reversal with fresh DME-FBS. Data points represent the averages of triplicate determinations ± SEM.

of exposure of the medium to source cells (Fig. 7). The data are expressed as percent inhibition of thymidine incorporation in CM-treated cultures compared to UCM-treated cultures (percent inhibition = 100% − (CM/UCM) × 100).

Growth inhibitory activity was observed in CM prepared from all source cell densities. Although the level of activity was highest in CM collected from cells arrested at the saturation density, this activity was clearly observable even when CM was prepared from source cells at 4×10^3 cells/cm^2, one-tenth of the saturation density. When the volume of the medium conditioned by a constant number of source cells was varied, the inhibitory activity decreased with increasing volume of medium. This dilution effect was substantiated by varying the CM volume and source cell density proportionally. Cultures at the low cell density used to condition a small volume of medium yielded as much inhibition as cultures at higher densities used to condition larger volumes of medium. Therefore, a minimum number of cells is required to condition a unit volume of medium.

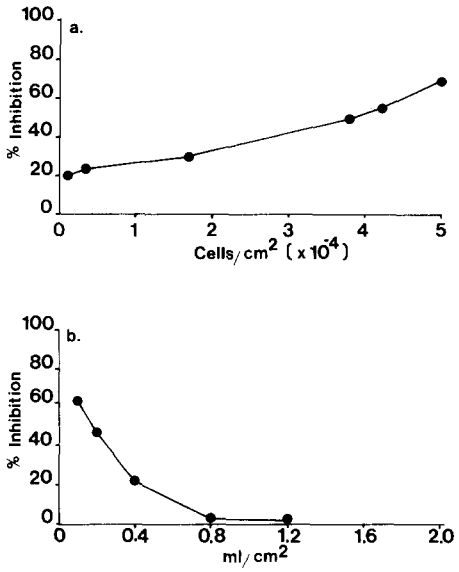

FIGURE 6 (*a*) The effect of varying the density of source cells on the inhibitory activity of the CM. Parallel cultures of 3T3 cells in plastic tissue culture flasks (25 cm^2 growth surface) were used to condition a constant volume (2.5 ml) of DME-FBS. (*b*) The effect of varying the volume of the medium conditioned by the same number of source cells on the inhibitory activity of the CM. Parallel cultures of 3T3 cells at the same density (4×10^4 cells/cm^2) in plastic tissue culture dishes (10 cm^2 growth surface) were used to condition different volumes of DME-FBS; the parameter expressed on the abscissa of this graph represents the volume of medium added per unit area of growth surface. The inhibitory activity of the CM was tested on target cells using the following conditions: target cells at 5×10^3 cells/cm^2, 24 h of exposure to CM, 3-h pulse with [^{3}H]thymidine (1 μCi/culture). Data points represent the averages of measurements on duplicate cultures and are expressed as percent inhibition as defined in the text.

TABLE III

Effect of Cell Number, Cell Density, and the Volume of the Medium on the Accumulation of Inhibitory Activity in CM

Cell No.	Growth area	Volume of CM	Cell density	Cell No.*/ volume of CM	Inhibition‡
	cm^2	*ml*	$cells/cm^2$		%
8.7×10^5	25	2.5	3.5×10^4	3.5×10^5	44.3
2.0×10^6	75	3.7	2.7×10^4	5.4×10^5	70.7
1.2×10^6	75	1.9	1.6×10^4	6.3×10^5	72.4
1.3×10^6	150	1.9	8.9×10^3	6.8×10^5	74.5

* This ratio reflects the number of cells used to condition 1 ml of medium.

‡ Data points represent the averages of measurements on duplicate cultures and are expressed as percent inhibition as defined in the text.

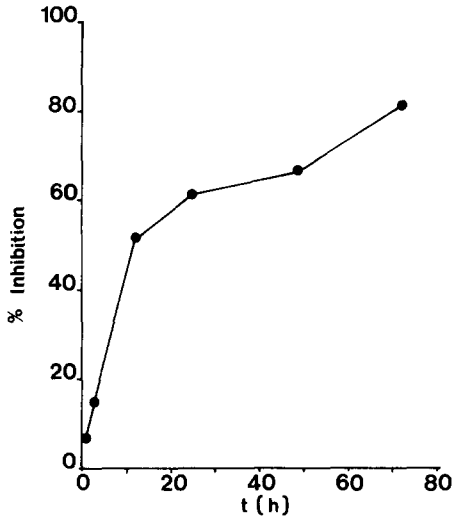

FIGURE 7 The effect of varying the length of exposure of DME-FBS to source cells on the accumulation of inhibitory activity in the CM. 3T3 cells (5×10^4 cells/cm^2) cultured in plastic tissue culture flasks (25 cm^2 growth surface) were used to condition a constant volume (2.5 ml) of DME-FBS for different lengths of time and the resulting CM was tested on target cells using the following conditions: target cells at 5×10^3 cells/cm^2, 24 h of exposure to CM, and 3-h pulse with [^{3}H]thymidine (1 μCi/culture). Data points represent the averages of measurements on duplicate cultures and are expressed as percent inhibition as defined in the text.

These results also indicate that cell-to-cell contact was not required for the generation of the inhibitory activity and that the level of the activity possibly reflected the concentration of the molecular species in the medium.

The effect of variations in the length of exposure of the medium to source cells was studied using confluent source cell cultures (5×10^4 cells/cm^2). A rapid increase in inhibitory activity (from 6 to 52%) was observed during the first 12 h of exposure (Fig. 7). The increase in the activity was minimal over the next 20 h.

Target Cell Parameters

In this series of experiments, the effect of varying conditions of the target cells during the period of exposure to CM was tested using a single preparation of CM. The effect of variation in the density of the target cells on the inhibition by CM is shown in Fig. 8. At target cell densities of 5×10^3 cells/cm^2 or above, the inhibitory activity of CM was consistently observed at the 60–70% level. In contrast, when the density of the target cells was below 2.5×10^3 cells/cm^2, there was a dramatic decrease in the potency of CM to inhibit 3T3 cell growth. Therefore, a key finding of our present studies is that a minimum target cell density may be required before the effect of CM can be observed.

We have also tested the effect of CM prepared from 3T3 source cells on a variety of other target cells. On the basis of the data obtained so far (Table IV), the following generalizations can be made. First, the normal cells 3T3, Nil-2, and chicken embryo fibroblasts showed the greatest sensitivity to inhibition by CM. Autoradiographic analysis of Nil cells treated with CM and UCM showed that the difference in [^{3}H]thymidine incorporation was reflected by a decrease in the percent of radioactively labeled cells in the CM cultures. Second, the SV 40 and polyoma virus-transformed fibroblasts showed much less inhibition, when tested with the same CM, than the normal counterparts. This was particularly true at high target cell densities. Third, of the two nonfibroblast lines tested, the epithelial line LCC-RK$_1$ derived from

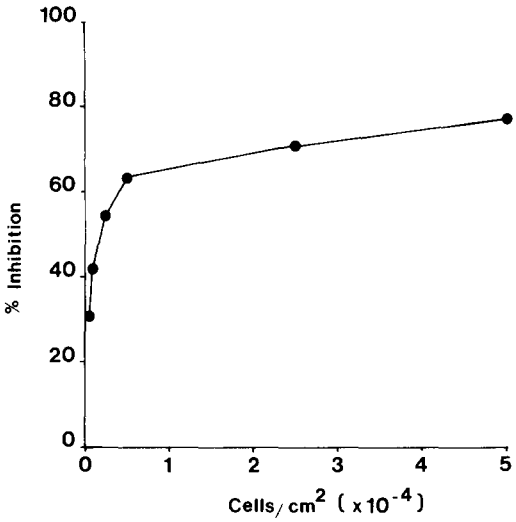

FIGURE 8 The effect of varying the density of target cells on the inhibitory activity of CM. CM was prepared using 5×10^5 source cells per milliliter of DME-FBS and 24 h of exposure time; the inhibitory activity was tested by treating target cells at different densities with the CM for 24 h followed by a 3-h pulse with [^{3}H]thymidine (1 μCi/culture). Data points represent the averages of measurements on duplicate cultures and are expressed as percent inhibition as defined in the text.

TABLE IV

Cellular Specificity of the Effect of CM on the Inhibition of [^{3}H]Thymidine Incorporation

Target cells*	Inhibition‡	
	2×10^4 Target cells/cm^2	5×10^4 Target cells/cm^2
	%	
3T3	54.2	50.7
Nil-2	60.5	59.9
CEF	47.8	40.4
SV 3T3	14.6	44.9
py Nil-2	7.7	27.4
LCC-RK$_1$	32.0	2.3
N4TG3	13.8	29.1

* The target cells used are: 3T3, a cell line derived from mouse embryo fibroblasts; Nil-2, a cell line derived from hamster embryo fibroblasts; CEF, secondary cultures of chicken embryo fibroblasts; SV 3T3, SV 40 virus-transformed 3T3 cells; py Nil-2, polyoma virus-transformed Nil cells; LCC-RK$_1$, a cell line derived from rabbit kidney cells; N4TG3, a neuroblastoma cell line.

‡ Data points represent the averages of measurements on duplicate cultures and are expressed as percent inhibition as defined in the text.

rabbit kidney cells and the neuroblastoma line N4TG3, both showed weaker inhibition by CM than the normal fibroblasts. As was observed with transformed fibroblasts, the neuroblastoma line also showed much less inhibition at the high target cell density. The results suggest that the effect of CM prepared from 3T3 source cells exhibit some cellular specificity with respect to the targets.

Conversely, experiments in which 3T3 cells served as target cells for testing the growth inhibitory activity of conditioned media prepared from other source cells were also performed. When DME-FBS were exposed to Nil-2, polyoma-transformed Nil-2, and SV 40-transformed 3T3 cells, the resulting conditioned media inhibited DNA synthesis in 3T3 target cells when compared to unconditioned media. We have not carried out further characterization of the inhibitory activities from these various source cells.

Evidence for Soluble Inhibitory Factors in CM

When target cells were treated with CM for more than 40–50 h, the level of [^{3}H]thymidine incorporation begins to rise (Fig. 2). This increase was paralleled by a similar rise in the number of cells in CM-treated cultures (Fig. 4). As a result, the degree of inhibition, as measured by comparing the levels of [^{3}H]thymidine incorporation in CM- and UCM-treated cultures, showed a gradual but consistent decrease (Fig. 9).

Although the details of this phenomenon are not well understood, one possible explanation is that an inhibitor present in the CM may be losing its effect due to denaturation or degradation. Experiments were performed in which CM-treated cultures were refed, after removal of old CM, with freshly prepared CM every 24 h. The inhibition was increased with the addition of newly replenished CM (Fig. 9). These data would argue against the contention that the failure of CM to support the proliferation of target cells was due to depletion of medium components because one would not expect the restoration of these components by long periods of exposure to target cells.

This conclusion is further supported by the following series of experiments using CM (SF). When 3T3 target cells were treated with CM (SF), DNA synthesis was significantly inhibited as compared to parallel cultures treated with UCM (SF) (Table V). In autoradiographic assays, the fractions of labeled cells in UCM (SF)- and CM (SF)-treated

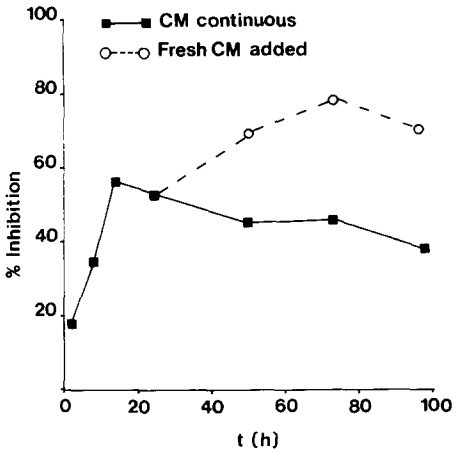

FIGURE 9 The effect of varying the time of exposure of CM to the target cells on the inhibitory activity. CM was prepared using 5×10^5 source cells per milliliter of DME-FBS and 24 h of exposure time; the inhibitory activity was tested by treating target cells (5×10^3 cells/cm^2) for various times followed by a 3-h pulse with [^{3}H]thymidine (1 μCi/culture). In one series of cultures, the target cells were exposed continuously to the CM added initially (■); in another series of cultures, the CM was replaced every 24 h with freshly prepared CM (○). Data points represent the averages of measurements on duplicate cultures and are expressed as percent inhibition as defined in the text.

cultures were 57 and 42%, respectively. These results indicate that the inhibitory activity can be collected in serum-free medium and can be detected despite the presence of freshly added serum.

The availability of the growth inhibitory activity in CM (SF) has also allowed us to characterize its molecular properties. The inhibitory activity can be detected in the precipitated fraction of CM (SF) after treatment with ammonium sulfate at 70% saturation (Table V). Little or no activity was found in the supernatant fraction. Moreover, similar treatment of UCM (SF) did not show any partitioning of growth-supporting functions. The ability to precipitate inhibitory components from CM (SF) and to redissolve them in fresh DME demonstrate that the activity in CM is not due to depletion of synthetic components in the DME.

It should be noted that to remove ammonium sulfate, both the supernatant and precipitate fractions were first dialyzed against large volumes of PBS and DME before they were combined with fetal bovine serum and tested on target cells. We have found, however, that dialysis of the CM (SF) resulted in the loss of inhibitory activity (Table V). Although the possibility that the inhibitory factor may be adsorbed on the dialysis membrane has not been eliminated, the present result does suggest that the inhibitor may be dialyzable and therefore,

TABLE V

Effect of Ammonium Sulfate Fractionation and Dialysis on the Growth Inhibitory Activity in CM (SF)

Conditions	UCM	CM	Inhibition
	cpm		*%*
DME-FBS	24,300 ± 2,000	13,000 ± 600	42.8
DME	25,300 ± 1,600	19,900 ± 1,000	21.2*
DME, $(NH_4)_2SO_4$ supernate, dialyzed	26,500 ± 2,600	24,100 ± 2,300	9.0
DME, $(NH_4)_2SO_4$ precipitate, dialyzed	24,100 ± 2,100	15,900 ± 500	34.2*
DME, dialyzed	21,100 ± 1,100	22,800 ± 2,400	—

Ammonium sulfate was added to 70% saturation. The experimental procedures used in ammonium sulfate fractionation and dialysis are described in Materials and Methods. The data represent the level of [^{3}H]thymidine incorporation in 3T3 target cells (5×10^3 cells/cm^2) exposed to UCM and CM for 24 h. The data are expressed as averages of triplicate determinations ± SEM. The inhibitory activity is expressed as percent inhibition as defined in the text.

* The efficiency of the isolation procedure can best be judged by a comparison of the specific activities of the original and fractionated material (specific activity = percent inhibition per milligram of protein in CM). The specific activities of unfractionated CM and the dialyzed ammonium sulfate precipitate fraction were 66 and 10, respectively.

may be a substance of low molecular weight. This loss of activity on dialysis may account for the decreased specific activity of the dialyzed ammonium sulfate precipitate fraction (Table V).

To circumvent the problem of activity loss during dialysis, we have fractionated the ammonium sulfate precipitate on a column of Sephadex G-15 equilibrated with DME. The inhibitory activity was associated with fractions corresponding to the void volume of the column (Fig. 10). There was a direct correlation between the inhibitory activity and the protein content of the fractions. More importantly, the corresponding fractions from a parallel experiment using UCM (SF) did not show any inhibitory activity. Autoradiographic experiments showed that the percent of labeled cells in target cultures treated with the peak fractions derived from CM (SF) and UCM (SF) were 21 and 51%, respectively. We have also ascertained that the inhibition by the fractionated CM (SF) material was reversible, indicating that we have not concentrated a toxic factor in our purification procedure. Finally, to establish the concentration range within which a linear response of the inhibition of thymidine incorporation assay can be obtained, we have diluted the fractionated CM (SF) material with UCM (SF). The assay was linearly dependent on the concentration of the inhibitory fraction up to the highest concentration available (~60% inhibition). All of these results appear to argue against the depletion of medium hypothesis, both in terms of serum factors as well as in the defined components of DME. Moreover, the data suggest that this system may be developed for the purification and characterization of the putative growth inhibitory factors.

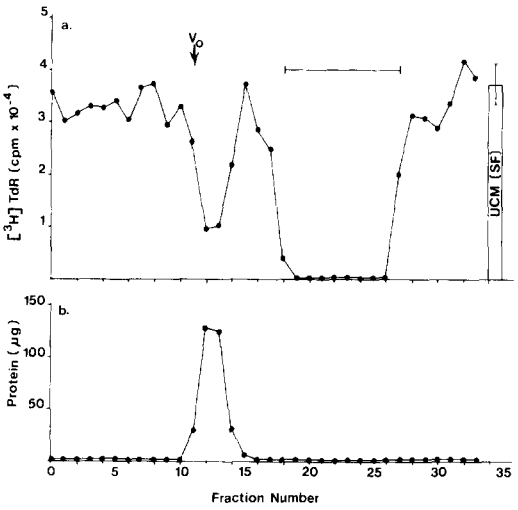

FIGURE 10 Chromatography of the ammonium sulfate precipitate fraction of CM (SF) on a column (1 × 60 cm) of Sephadex G-15 equilibrated with DME. The ammonium sulfate (70% saturation) precipitate was redissolved in 1 ml of DME and applied to the column. Fractions of 1 ml were collected and assayed for (*a*) inhibition of [^{3}H]thymidine (*[^{3}H]TdR*) incorporation in target cells and (*b*) protein content. The fraction corresponding to the void volume of the column is indicated by the V_0 symbol. The horizontal bar represents the fractions which lysed the target cells resulting in no incorporation of [^{3}H]thymidine; these fractions were found to contain ammonium sulfate. The vertical bar labeled UCM (SF) represents the average level of [^{3}H]thymidine incorporation (± SEM) in target cells treated with fractions obtained from chromatography of serum-free UCM; the fractions corresponding to those indicated by the horizontal bar also showed no incorporation and were excluded in the calculation of this average. The specific activities (percent inhibition per milligram of protein in CM) of the original CM and the peak fraction of the column were 36 and 41, respectively.

DISCUSSION

The mouse fibroblast line 3T3 exhibits a form of growth control in vitro in that it reaches only a very low saturation density and can remain for long periods of time in a viable but nondividing state. On the basis of analyses performed in a number of laboratories, the mechanisms responsible for this phenomenon of density-dependent inhibition of growth can be arbitrarily classified into three major categories (Fig. 11): (*a*) cell-to-cell contact mediated growth arrest; (*b*) depletion of growth factors in the medium and cessation of cell proliferation; and (*c*) accumulation of inhibitory products in cultures of high cell density.

Although these three mechanisms are not necessarily mutually exclusive, the demonstration of a growth inhibitory activity in CM reported in our present studies suggests that the observed growth regulation at high cell densities may be partly mediated by soluble inhibitory factors (hypothesis III, Fig. 11). Our results suggest that the growth inhibitory factor in CM prepared and tested in the 3T3 system has the following key features: (*a*) the inhibitory activity can be demonstrated using three different assays of cellular DNA synthesis and proliferation; (*b*) it is not cytotoxic and its effects on cell growth are reversible; (*c*) the inhibitory activity can be accumulated in the medium before the onset of extensive cell-to-cell contact; (*d*) the inhibitor has a more pronounced effect on target

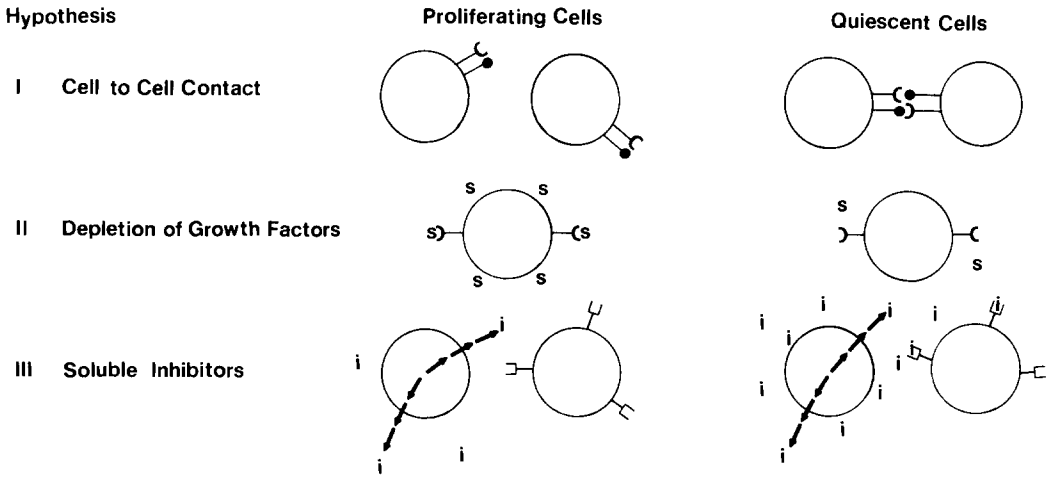

FIGURE 11 Schematic representation of three hypotheses to account for density-dependent inhibition of growth in cultured fibroblasts. The symbols s and i denote growth stimulatory factors and growth inhibitory factors, respectively.

cells at high density than on cells at lower density; (*e*) the activity can be collected in the absence of serum and can be demonstrated despite the presence of freshly added serum; (*f*) the activity is decreased upon prolonged exposure to target cell cultures; and (*g*) the inhibitory factor can be concentrated and fractionated by precipitation and gel filtration, respectively. We shall consider below the interpretation of these facts in relation to the data accumulated in support of the three major hypotheses on growth control in cultured cells.

Several previous lines of evidence have suggested that medium conditioned by exposure to density-inhibited 3T3 fibroblasts may contain soluble factors that inhibit cell growth and division (hypothesis III, Fig. 11) (9, 23, 24, 35). In addition to the 3T3 cell system, growth inhibitory factors in medium conditioned by exposure to density-inhibited cells have also been studied in the WI-38 (13) and BSC (10) cell systems. The present study extends the work of Harel et al. (9), as well as those of Rheinwald and Green (23, 24), in three major respects. First, we have provided confirmation of their original observations using assays of cellular DNA synthesis. Second, we have obtained evidence which suggests that the presence of an inhibitory factor rather than depletion of medium components may be responsible for the observed activity. Finally, we have reported the interesting finding that a minimal target cell density may be required before the effect of CM can be detected, thus offering a possible mechanism for density-dependent growth control. Obviously, these analyses are still limited by the lack of purified preparations of the putative inhibitors and must await progress on their molecular characterization.

Holley and Kiernan (11) have proposed that depletion of growth nutrients, particularly the serum factors, may be responsible for inhibition of fibroblast proliferation at high cell densities (hypothesis II, Fig. 11). In support of this notion, they have shown that the final cell density of 3T3 cells in a confluent culture was directly proportional to the concentration of serum in the medium. It has also been shown that used medium could be restored by dialysis against fresh medium and by addition of low molecular weight constituents (27). Stoker (28) and Stoker and Piggott (29) have proposed that the entry of essential growth factors into 3T3 fibroblasts grown to high cell densities may be limited by a diffusion boundary layer surrounding the cells. This represents a local depletion of the required growth stimulators in mediating cessation of cell division.

The fact that we can observe inhibitory activity in CM (SF), even though it is tested in the presence of freshly added serum, indicates that at least part of the growth cessation was due to the presence of some inhibitory substance. More importantly, the fact that we can concentrate the inhibitory activity by ammonium sulfate precipitation and reconstitute it in fresh DME by gel filtration strongly suggests that the failure of CM (SF) to support cell division of target 3T3 cultures cannot be ascribed

to a depletion of growth nutrients. If depletion of nutrients from CM were responsible for its activity, it would be hard to conceive mechanisms that would allow the retention of inhibitory activity after precipitation and reconstitution in fresh medium. This conclusion is further supported by the observation that the effect of CM was diminished on prolonged exposure to target cells, suggesting that the inhibitor may be denatured or degraded.

That cell-to-cell contact was an important requirement in the mechanism of growth regulation (31, 32), in analogy to the phenomenon of contact-mediated inhibition of cell movement (1), was first suggested by the observations that confluent 3T3 cultures showed little or no growth, while subconfluent ones proliferated rapidly. It has also been reported that DNA synthesis in 3T3 cells grown in colonies occurred predominantly at the periphery of the colony, while the cells at the center did not incorporate [^{3}H]thymidine (7). In contrast, CHL-1 cells, which do not exhibit density-dependent inhibition of growth, revealed an even distribution of [^{3}H]thymidine incorporation in colony cultures. The contact inhibition of growth hypothesis (hypothesis I, Fig. 11) is also supported by the wound healing experiments (5, 6, 32) in which rapid cell growth and division was observed when a confluent monolayer of 3T3 cells was "wounded" by removal of a strip of the cell layer. Moreover, sparse 3T3 cells continued their growth when they are separated from density-inhibited cells by a membrane filter (26). These experiments argue against the action of a diffusable inhibitor because both the proliferating as well as the quiescent cells were bathed in the same medium.

To reconcile these well-documented observations with our present findings, one would have to postulate that both contact-mediated and soluble inhibitor mediated mechanisms may be operative in density-dependent regulation of cell growth (hypotheses I and III, Fig. 11). The wound healing and membrane filter experiments do not rule out the presence of soluble factors, considering the fact that the volume of the medium used may have diluted out any effect of the inhibitory factor. It should also be noted that in our CM-treated cultures, there is always a finite percentage of cells undergoing DNA synthesis and cell division. Therefore, one would expect that wound healing could take place even in the presence of CM. One key analysis that remains to be performed is to compare the rate with which a wound is repaired when it is continuously flushed with CM or with UCM.

More recently, Whittenberger and Glaser (33) and Whittenberger et al. (34) showed that a plasma membrane-enriched fraction of 3T3 cells inhibited DNA synthesis in the same cells but not in their SV 40 virus-transformed counterparts. Natraj and Datta (18) have also shown that an inhibitor of DNA synthesis can be extracted from 3T3 cells by treatment with 0.2 M urea in PBS. The inhibitory activity of this factor was abolished by glycosylation with *N*-acetyl-D-glucosamine; the inactive factor could in turn be converted to the active form by treatment with *N*-acetyl-β-D-glucosaminidase. It was suggested that these surface membrane molecules which inhibit normal cell proliferation may be the same molecules that are responsible for contact-dependent growth regulation.

The relationship between the growth inhibitory activity in the CM of our present studies and the similar activity observed in the membrane fractions has not been determined. It is possible that the same molecule can exert its effects both anchored on the cell surface or released into the medium (hypotheses I and III, Fig. 11). Comparisons of the results from these two parallel lines of experiments on cell-bound and soluble forms of growth inhibitory factors should facilitate their isolation and characterization as well as contribute to our understanding on the mechanisms of growth control.

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