A DIFFUSIBLE FACTOR WHICH SUSTAINS CONTACT INHIBITION OF REPLICATION

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ABSTRACT

Confluent 3T3 cultures make and release into the medium a diffusible factor which sustains the state of contact inhibition of replication. Evidence is given that the factor is a specific and reversible inhibitor of the RNA synthesis which precedes cellular replication.

INTRODUCTION

The study of colony formation of cultured mammalian cells (7) demonstrates that contact between cells with the property of contact inhibition of replication initiates a sequence of intracellular events which interfere with the unlimited proliferation of the central cells in the colony. This phenomenon takes place at cell densities which do not produce medium depletion or modification; hence, it lends further support to the conclusion reached in other laboratories (13, 20) that inhibition of replication probably is not caused by humoral factors wich are released into the medium by some cells to inhibit replication of their neighbors. However, the intracellular phenomena still might be mediated by a diffusible factor. One hypothesis is that cell contact induces or activates the internal release of an inhibitor of macromolecular synthesis, which in turn produces a cessation of replication.

This hypothesis was tested by looking for a diffusible inhibitor of cell replication released into the media by contact inhibited cells. The experimental results to be described demonstrate the presence of such a diffusible inhibitor which sustains contact inhibition of replication in the mouse cell line 3T3. This inhibitor is reversible and acts specifically to stop ribonucleic acid synthesis of contact inhibited cells.

MATERIALS AND METHODS

Cells and Cultivation

The origin and growth characteristics of the cell line 3T3 have been described in detail previously (7, 18, 19). Both 3T3 cells and a simian virus-transformed line of 3T3 cells called SV-61 cells were kindly supplied by Dr. Howard Green (Department of Pathology, New York University School of Medicine, New York). The maintenance medium for these cells consisted of Eagle's basal medium (5) with four times the usual concentration of vitamins, amino acids, and glucose, and Earle's balanced salt solution, 10% calf serum, and antibiotics. HeLa and CHL-1 cells were maintained by methods previously described (7–10).

Assay Procedures

The cultures used in experiments were started in 35-mm plastic Petri dishes at an inoculum of 1×10^5 cells per dish. The medium was changed twice per week until the cultures attained confluency. In this medium a cell density of 4×10^4 cells/cm^2 was obtained for use in assay experiments.

RNA synthesis was measured by preincubating the assay cultures in their respective experimental environments for 30 min and then by pulse labeling them for 15 min by adding 0.05 ml of 4.5 μc/ml uridine-2-^{14}C (specific activity greater than 20 mc/

mmole). The dishes were then washed three times with 2-ml portions of buffered saline solution (10). The monolayer of cells was dissolved with 0.5% recrystallized sodium lauryl sulfate solution in Tris-Cl buffer (pH 7.4) and precipitated with an equal volume of cold 10% trichloroacetic acid. The precipitates were collected on Millipore filters, washed four times with 5 ml of cold 5% trichloroacetic acid, dried under an infrared lamp, and counted on a gas flow counter.

DNA synthesis was measured either by pulse labeling or by continuous labeling of the test cultures by adding 0.05 ml of 10 μc/ml thymidine-2-^{14}C (specific activity 53 mc/mmole). DNA samples were collected and counted in the same way as the aforementioned RNA samples.

The rate of mitosis was determined by removing test dishes, washing them free of medium, and fixing them for 1 hr with a 10% formalin solution. After the dishes had been washed free of fixative, a solution of hematoxylin was added to each dish and allowed to stain overnight. Excess stain was washed off, and the dishes were rinsed with a weak NH_4OH solution and air-dried. Cover glasses were mounted on the dishes with Permount (Fisher Scientific Company, Pittsburgh, Pa.), and the cells were examined under a low power microscope. The mitotic figures were counted, and their fraction of the total cells in at least 50 fields of two or more dishes was determined.

RESULTS

Effect of Cell Density on DNA Synthesis

The rate of DNA synthesis as a function of cell density for 3T3 and CHL-1 cells is shown in Fig. 1. These findings agree with the results reported by several laboratories (13, 20) which show a drastic decrease in the DNA synthesis of 3T3 cells in this media at densities greater than 2×10^4 cells/cm^2. Microsopic examination revealed that the cells began to come into contact with each other at this density. At a density of 4×10^4 cells/cm^2, the culture was confluent, and DNA synthesis was less than 10% of the maximum rate per cell found at lower cell densities. RNA synthesis, measured by 15 min of uridine-^{14}C incorporation, decreased along the same curve. By comparison, nucleic acid synthesis of CHL-1 cells decreased gradually, rather than sharply, with increased cell density, and at a much higher cell concentration, which suggests that a different

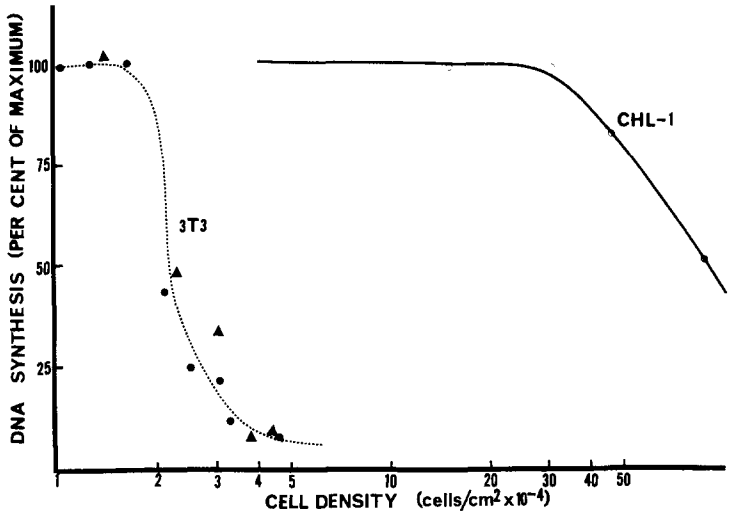

FIGURE 1 Comparison of DNA synthesis at various cell densities of 3T3 and CHL-1 cultures. Stock cultures were dispersed and counted, and the cells were plated at increasing concentrations. After 2 days, growth they were labeled for 24 hr with thymidine-^{14}C, and the cold, 5% trichloroacetic acid insoluble radioactivity was determined (●). In another experiment, cells were plated in replicate at 1×10^5 cells per dish and incubated. A set was labeled daily with thymidine-^{14}C for the radioactivity (▲), and duplicate dishes were trypsinized and counted to determine the cell number. In a third experiment CHL-1 cells were grown and labeled as described above (○). For each point the counting rate of DNA was divided by the cell density, and the per cent of the maximum rate was determined.

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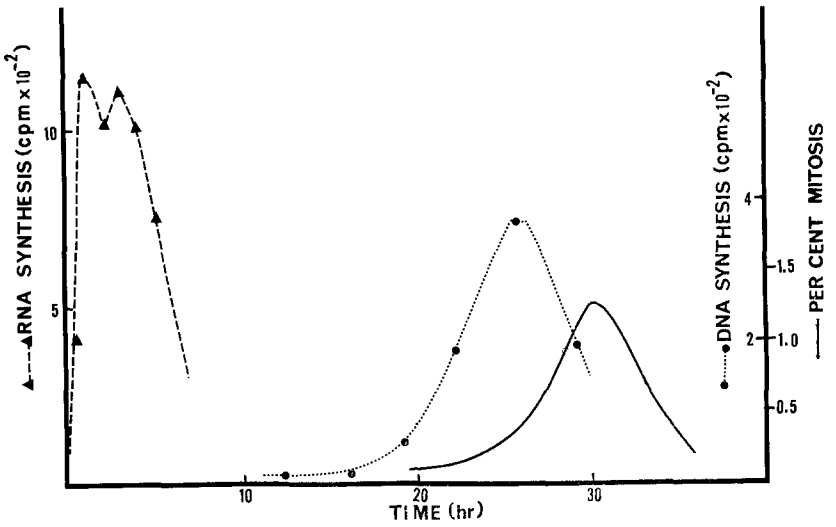

FIGURE 2 Time sequence of macromolecular synthesis after medium change on CI cultures of 3T3 cells. Cells were pulse labeled at various times after the medium had been changed to fresh medium by adding 0.05 ml of 4.5 μc/ml uridine-^{14}C or 10 μc/ml thymidine-^{14}C to replicate dishes, and the trichloroacetic acid-insoluble radioactivity was determined. In another set, mitotic figures were determined as described under Materials and Methods. All points are plotted as the difference in counts between CI medium samples and fresh serum medium samples. ▲, uridine-^{14}C RNA; ●, thymidine-^{14}C DNA; ○, mitosis.

phenomenon such as depletion of nutrients rather than the physical contact of cells was responsible for the decline.

Cultures of 3T3 cells were maintained in a contact inhibited (CI) state for many weeks without detectable increase in the cell population (19). The viability of such confluent CI cultures was the same as that of exponentially growing cultures, since in both cases the absolute plating efficiency was about 30% and the ability to concentrate vital stain was identical.

Effect of Medium Change on CI Cultures

When used medium of CI cultures was replaced by fresh serum medium, a wave of RNA synthesis was observed in all cells within 30 min followed by a wave of DNA synthesis and mitosis in a small fraction of the cells (see Fig. 2). This time sequence of events agrees with data published elsewhere (20). Since HeLa, CHL-1, and SV-61 cells—cell lines without contact inhibition—showed no such labeling pattern, the difference in incorporation of uridine-^{14}C after medium change was adopted as the basis for an assay system to measure the ability of media to sustain contact inhibition in confluent cultures of 3T3 cells.

Evidence for an Inhibitor

Recent studies on contact inhibition have attributed the wave of RNA and DNA synthesis and mitosis after medium change to stimulatory factors in fresh serum (20, 21). If serum activators were the sole factors responsible for the stimulation, then no response would be expected when serum-free medium was tested in the assay system. The average of 10 independent experiments, however, showed that serum-free media were able to stimulate 70% of the incorporation of uridine-^{14}C induced by fresh serum medium. Although cells will not undergo cell division in saline medium, if they are returned to the used medium after the wave of RNA synthesis in serum-free medium they are just as capable of DNA synthesis and cell division as cells in fresh medium. These results suggested that some substance was present in used medium of CI cultures which inhibited RNA synthesis.

Fresh medium incubated with CI cultures showed a loss of stimulatory ability as a function of incubation time. Maximum loss of stimulatory ability occurred after an incubation period of 3 days, when the inhibitory substance reached a saturating level. Post 3 day medium from con-

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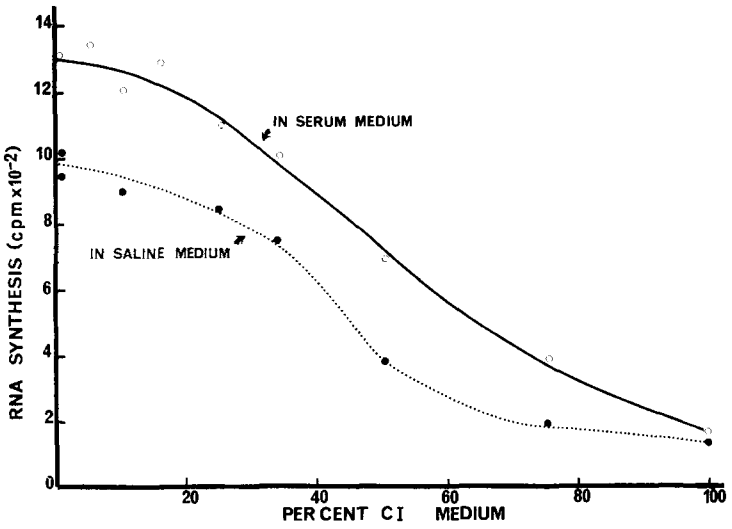

FIGURE 3 Rate of RNA synthesis in CI medium diluted with saline F and with fresh medium. Increased proportions of fresh serum medium and saline F medium were combined with CI medium, equilibrated with confluent cultures of 3T3 cells for 30 min, and assayed with uridine-^{14}C as described under Materials and Methods. Values for the dilution series of fresh serum medium with CI medium, ○; dilution series of saline F medium with CI medium, ●.

fluent 3T3 cultures was designated CI medium.

Two types of experiments suggested that the mitosis-inducing capacity of CI medium was as good as that of fresh medium. In the first place, the plating efficiency and colony growth rate of 3T3 cells in CI medium were consistently as high as or higher than those in fresh medium (7). Also, cells started at densities less than 2×10^4 cells/cm^2 always multiplied in CI medium until they reached the same confluent density as cells growing in fresh medium.

That the inhibitor substance could be diluted out of CI medium either by fresh serum medium or by saline medium is shown in Fig. 3. At all concentrations fresh serum medium was more effective than saline medium in diluting out the inhibitor, and it also stimulated the cells to a higher level of incorporation. The difference in incorporation between fresh serum medium and saline medium might be attributed to the action of a serum activator, for when serum was added to CI medium the maximum stimulation achieved was only 37% that of fresh medium even at an added serum concentration of 25%.

The results of dialysis experiments are shown in Table I. After exhaustive dialysis, the dialysate showed a level of stimulation of RNA synthesis almost as high as that of fresh serum medium.

TABLE I

Effect of Dialysis on CI Medium

Medium	Recovery dialysis*	Exhaustive dialysis‡
	cpm	*cpm*
Fresh serum medium control	3000	3000
CI medium control	400	400
Dialysate	685	2300
Dialysand	580	375
Fresh serum medium + dialysand of exhaustive dialysis	—	320

* For recovery dialysis, CI medium was dialyzed for 24 hr against an equal volume of fresh medium without serum.

‡ For exhaustive dialysis, samples of CI medium were dialyzed for 24 hr each against three changes of 100-fold greater volume of dialysand. To test the lyophilized concentrate of the dialysand, CI medium was dialyzed against distilled water. All experiments measured RNA synthesis as uridine-^{14}C incorporation into acid-insoluble material as described in Materials and Methods.

When equivalent amounts of the concentrated dialysand were added to fresh serum medium, the RNA synthesis in the fresh serum medium was reduced to the level of that in the CI medium.

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TABLE II

Cross-Tests of Media from 3T3 Cells with Media from HeLa and SV-61 Cells

Cell type	Medium*	
		cpm‡
3T3	Fresh serum medium	1800
	CI medium	400
	Used HeLa medium	1500
	Used SV-61 medium	1200
HeLa	Fresh serum medium	1800
	Used HeLa medium	1000
	CI medium	1100
SV-61	Fresh serum medium	1800
	Used SV-61 medium	1800
	CI medium	1700

* 3 day old medium from confluent 3T3 cultures and high density cultures of HeLa or SV-61 cells are designated, respectively, CI medium and used medium.

‡ Confluent cultures of 3T3 were incubated for 30 min in the used medium from high density dishes of HeLa or SV-61 cells, while HeLa or SV-61 cells were incubated in CI medium. Control dishes with fresh serum medium and their own used medium were tested simultaneously. All samples were pulse-labeled with uridine-^{14}C as described under Materials and Methods. The degree of inhibition of RNA synthesis was measured by incorporation of radioactivity into acid-insoluble material.

These results indicate that the inhibitor substance is of low molecular weight.

To rule out the possibility that the results were produced by a reduction in the specific activity of uridine-^{14}C or by the presence of a toxic substance, media from 3-day confluent dishes of 3T3, HeLa, and SV-61 cells were cross-tested against each cell line. Table II shows that the medium from the 3T3 culture inhibited its own RNA synthesis fivefold, but that this medium had little or no effect on HeLa and SV-61 cultures. The medium from HeLa and SV-61 cultures had no inhibitory effect on 3T3 cells or on HeLa or SV-61 cells.

Site of Inhibition

When the inhibitor's effect on RNA synthesis was tested by incubating CI cutures in fresh serum medium before changing to CI medium, the RNA synthesis stimulated by fresh serum medium was halted within 20 min of the return to CI medium. To test the effect of the inhibitor on DNA synthesis, the wave of RNA synthesis shown in Fig. 2 was allowed to take place in fresh serum medium before it was changed back to CI medium. Cultures completing the wave of RNA synthesis in the fresh serum medium also incorporated thymidine-^{14}C, regardless of whether they were later left in fresh serum medium or changed to CI medium. CI cultures incubating for increasing lengths of time in fresh serum medium before being changed back to CI medium demonstrated that cultures which synthesized RNA for less than 5 hr were unable to follow through the wave of DNA synthesis.

TABLE III

Tests on the Nature of the Inhibitor Substance

Test medium	Response*
1. Fresh medium	Control
2. CI medium	+
3. Used SV-61 medium	−
4. Used HeLa medium	−
5. CI medium heated to 56°, 70°, and 100°, 15 min	+
6. CI medium frozen and thawed twice	+
7. CI medium stored at 4° and −20°C, 1 wk	+
8. Eluent of CI medium from Sephadex G25 and G75 in mol wt 400–900 range	+
9. Eluent of CI medium from Sephadex G25 and G75 in mol wt 2000	−
10. CI medium with 2, 10, and 20% bovine fetal serum	+
11. CI medium with 2, 10, and 20% calf serum	+
12. CI medium with 10% horse serum	+
13. Fresh medium incubated 4 days, 37°C	−
14. Fresh medium from 16 hr incubation with CI cells and incubated 4 days, 37°C, without cells	−

* A positive response indicating presence of inhibitor is scored if the rate of uridine-^{14}C incorporation in the assay system described in Materials and Methods was less than 25% that of the fresh medium control.

Nature of the Inhibitor

Table III summarizes the results of a series of tests on the stability, size, and composition of the inhibitor. The inhibitor appears to be a stable substance of low molecular weight and could be

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removed from CI medium by dialysis to rejuvenate the medium. The dialysand, concentrated by lyophilization, was as inhibitory as CI medium, and addition of it to fresh serum medium made the medium inhibitory for 3T3 cultures but not for HeLa cells.

Fucose has been described as a cell surface-reactive substance which affects contact inhibition (3). Tests on the ability of fucose to produce the inhibition of RNA synthesis in this system were unsuccessful up to concentrations of 12.5 mg/ml. Analysis of the dialysand by paper chromatography (4) did not reveal a significant level of fucose.

Calf serum is known to contain the enzyme diamine oxidase which converts certain polyamines, such as spermine, to acrolein, an extremely cytotoxic substance (1). The tests shown on Table III, in which various serum concentrations and serum types were employed and in which incubation of medium without cells was tested, do not support the idea of conversion, by serum enzymes, of medium components to cytotoxic substances. Differential inhibition of RNA synthesis of 3T3 cells without comparable effects on HeLa or SV-61 cells was used as a control of general cytotoxic effects produced during purification or treatment.

A fractionation system which showed promise for purification and identification of the inhibitor substance was that utilized by Raina and Cohen in the study of polyamines (16). Initial tests indicated that more than a 100-fold increase in specific activity of the inhibitor in CI medium was achieved through the butanol extraction with almost a 50% recovery. Control extracts of medium from SV-61 cultures did not produce an inhibitory activity.

DISCUSSION

Probably the most interesting conclusion that can be drawn from the aforementioned results is that there exists a diffusible factor in the 3T3 cell which plays a role in control of macromolecular synthesis and, hence, in cell replication. Since DNA synthesis and mitosis occurred in the medium reversal experiments after the cultures were returned to the inhibitory medium following the wave of RNA synthesis, we can postulate that the regulation is mediated through the control of synthesis of RNA specifically.

The specific requirement of complete cell-to-cell contact before activation of the inhibitor was previously demonstrated in the clonal growth experiments (7). This requirement is again emphasized in the present results shown in Fig. 1 and by the fact that the routine plating efficiency in CI media was always as good as or better than that in fresh media. Hence, although serum concentration may be operative in regulating the cell density at which the contact inhibitory stimulus takes place (11), and although it may act in binding inhibitory molecules in the medium, it cannot be used to prevent the production of CI medium by 3T3 cells. One possible explanation of the paradoxical finding that cell contact is necessary before the extracellular inhibitor becomes effective is that physical contact of cells which have the property of contact inhibition triggers a change in their permeability and, thereby, permits release and entry of the diffusible inhibitor.

Many previous studies of factors in mammalian cell replication have emphasized serum macromolecular components such as glycoproteins (14), fetuin (6, 15), or alphaglobulins (12). While these factors are all stimulatory for in vitro cell multiplication, the present study emphasizes a regulation through the inhibitory effect of a diffusible substance. A system combining the serum stimulatory factor described by Todaro et al. (21) and the present inhibitory factor permits speculation on balance mechanisms for the description of contact inhibition of replication.

Recently diffusible substances native to cells have been studied by Raina et al. (17) in the regulation of RNA synthesis in *Escherichia coli*. The polyamines, putrescine and spermidine, have been portrayed as the natural control system for RNA metabolism. It is not known whether the polyamines act at the level of transcription or produce instability following transcription. There is evidence that putrescine may cause instability of preribosomal particles in *E. coli* (2), whereas spermidine seems to stabilize such particles. In the case of mammalian cells cultured in vitro, it has already been shown that contact inhibition of replication is accompanied by a loss of polysomes (13). We believe that the established aneuploidic cell line 3T3, which exhibits a high degree of contact inhibition of replication, may provide a very useful system for the study of the control of RNA synthesis by small molecules produced in culture, particularly when contrasted with other

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established cell lines or virus-transformed cultures.

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