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Serum-Stimulated Release of Cell Contacts and the Initiation of Growth in Contact-Inhibited Chick Fibroblasts

(monolayers/rate of cell movement/time-lapse cinematography)

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ABSTRACT **Increased serum concentration in the medium of a confluent culture increases the overlapping of cells and the average rate of cell movement. The relationship between these two serum activities and serum release of growth inhibition was studied. The increase in overlaps does not appear to be directly related to release of growth since it occurs well after growth has been initiated. The increased rate of cell movement occurred immediately after the addition of serum and was quantitatively proportional to the stimulation of DNA synthesis over a range of serum concentrations, implying that both movement and growth are released by a common serum activity. Direct microscopic observation and time-lapse films reveal a reduction in cell contacts concurrent with the increase in cell movement. Experiments showed that cell movement at low cell densities, where cell contacts are minimal, was rapid in low serum concentration and was not stimulated by increasing the serum concentration. This suggests that the serum effect on cell movement involves cell contacts and is due to release of cell contacts in the confluent monolayer. The primary action of serum may be the disruption of adhesive cell contacts.**

The cessation of growth and the formation of a monolayer by normal cells as they reach confluence in a tissue culture dish are expressions of normal growth controls that are lost by malignant cells (1, 2). The cessation of growth, which occurs at characteristic densities for each cell line, seems to depend on cell to cell contact or at least on close proximity (2, 3). Although a definite role for cell contact has never been established in growth control (4, 5), the limitation of growth has usually been called "contact inhibition of growth". Contact inhibition applies more precisely to the cessation of forward movement upon contact between the surfaces of two normal cells moving toward each other (6). This contact inhibition of movement prevents the cells from moving over one another and appears to be related to the formation of a monolayer by normal cells growing in a culture dish. Contact inhibition of growth and contact inhibition of movement may be related, since cells showing an effective growth control form monolayers, while those showing little growth control pile up into multilayers (2).

The experiments we describe in this paper were designed to examine the relationships between cell contact, contact inhibition of growth, and contact inhibition of movement. A convenient method of releasing growth inhibition in confluent cultures is to raise the serum concentration of the medium (7–9). Upon the addition of serum, cell growth is initiated and synthesis of rRNA is activated within 2 hr (7); DNA synthesis begins by 8–12 hr, mitosis starts by 20–30 hr (7, 10), and a new and higher saturation density is reached that depends on the amount of serum added (11). The serum alteration of the saturation density at which cells stop growing has suggested that cell contacts do not play a primary role in inhibiting growth (8, 9, 11,), since cells should come in contact at the same cell density, irrespective of the serum concentration. The evidence presented here suggests a compromising hypothesis that reconciles the action of serum with the idea that cell contacts play a primary role in growth control. We propose that the adhesiveness of the cells determines the saturation density at which cells can form the stable cell contacts necessary for growth limitation. Serum appears to reduce the adhesiveness of cells in the monolayer such that they pull apart from one another, move about, and begin to grow.

MATERIALS AND METHODS

Back skins removed from 9-day-old chick embryos were washed in Saline G, and incubated in 0.25% trypsin (Calbiochem A grade) in Saline G at 37°C for 15 min. The trypsinized skins were washed 3 times in Ham's F-12 medium NAB, Inc. with 1% chick serum and 1% horse serum added, and dispersed with a pasteur pipet. The dispersed cells were then filtered through 10-μm Nitex mesh, which removed most clusters of cells. The cell concentration was measured with a hemocytometer and the cells were plated at a density of 2×10^6 cells per 60-mm Falcon tissue culture dish or 30-ml Falcon tissue culture flask. 5 ml of medium with serum was added to each container and the cells were incubated at 37°C in a 5% CO_2 atmosphere. When cells were to be grown to confluence in 0.5% serum, the medium was changed at 24 hours to medium with 0.25% horse and 0.25% chick serum. Plates to be examined for overlapping nuclei were fixed in ethanol–acetic acid 3:1, washed with ethanol, and dried. Nuclei were stained with 0.5% toluidine Blue 0 in 0.2 M McIlvain's buffer at pH 4 for a few minutes. The plates were washed in distilled water and 95% ethanol, dried, and covered with immersion oil for examination.

Autoradiography

[^{3}H]thymidine (5 μCi/ml, 14 Ci/mmol) was added at the time of serum addition. The plates were incubated for 20 hr, fixed for 5 min in ethanol–acetic acid 3:1, washed 3 times in 5% trichloracetic acid, and washed once in absolute ethanol. After drying, the plates were coated with K5 Ilford emulsion and exposed for $2^1/_2$ days in a desiccator at 4°C. The plates

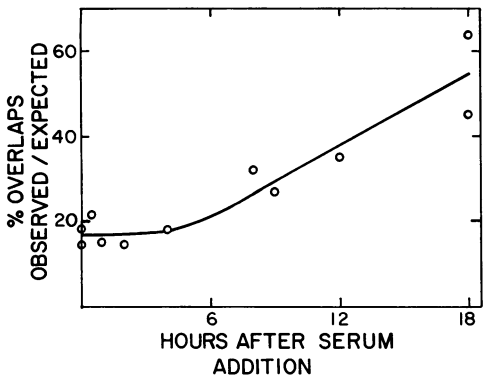

FIG. 1. Increase in overlapping nuclei in response to elevation of the serum concentration from 2 to 20%. The number of overlaps is expressed as the percentage of the overlaps expected if the cells were distributed randomly (for calculation of expected overlaps, see ref. 5). Plates were grown to confluence in 2% serum before the serum concentration was increased.

were developed in Kodak D-19, fixed in Kodak acid fixer, washed, and stained as described above.

Time-lapse cinematography

Bottles of cells were sealed, placed on a stage of an inverted phase microscope that was covered with a polyethylene bag, and heated to 37°C with an air-curtain incubator (Sage). The cells were photographed with Kodak Tri-X reversal film in an Ariflex-S camera. Rates of cell movement were measured by following nucleoli of individual cells with a stop-frame projector.

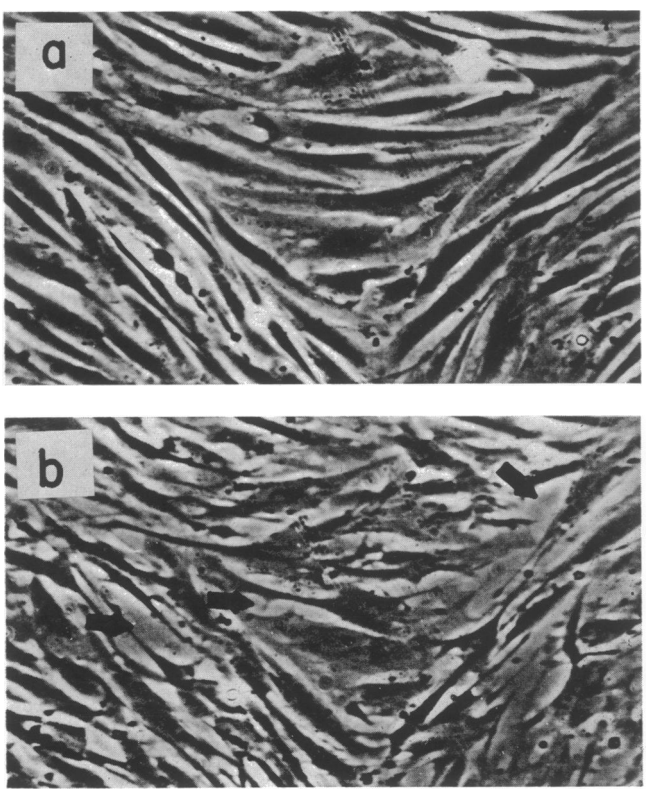

FIG. 2. Response of cells to an increase of the serum concentration from 2 to 20%. (*a*) A field of cells grown to confluence in 2% serum. (*b*) The same field 4 hr after the serum concentration was increased. The *arrows* on (*b*) point to areas of the petri plate not covered by cells. Such areas are not prominent on (*a*). ×238 magnification.

RESULTS

The serum effect on contact inhibition of movement

The possibility that serum released contact inhibition of movement as it released growth was examined. Since contact inhibition of movement restricts the movement of cells over one another, it can be determined from the incidence of overlapping nuclei (1). Overlapping nuclei were measured at various times after the serum concentration was increased to 20% (Fig. 1) in plates grown to confluence in 2% serum. Before the increase in serum concentration, the number of nuclear overlaps was only about 15% of those expected if the cells had been moving over one another randomly. Overlaps remained at this low level until 8 hr after the addition of serum, when they began to increase. Overlaps increased over the next several hours until cells were distributed almost randomly by 24–30 hr. Since growth initiation occurs by at least 2 hr, when RNA synthesis has increased (7), the increase in overlaps after 8 hr suggests that the release of contact inhibition of movement cannot be directly related to the primary event in the release of growth control, although some complex relationship may exist.

Release of cell contacts

During the examination of plates for overlapping nuclei, we noticed that contact relationships between cells changed soon after serum addition. The cells in higher serum concentrations were less well spread on the surface of the dish and did not adhere to each other as extensively (Fig. 2). When the changes in cell-contact relationships were followed by time-lapse cinematography, cells appeared to snap apart and began to move more rapidly. These observations indicate that serum reduces the strong adhesive cell to cell contact, which stretches the cells over the surface of the dish and restrains their movement.

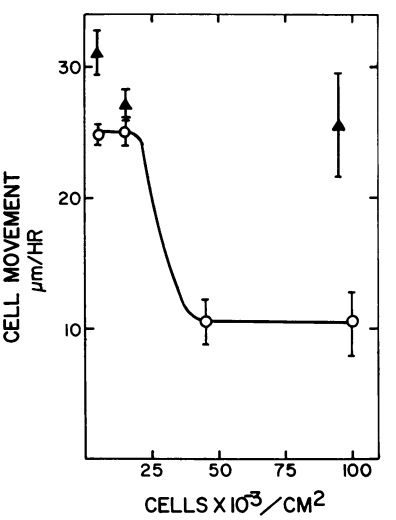

FIG. 3. The effect of cell population density on the average rate of cell movement. The rate of movement was measured in cultures of cells maintained in 2% serum for 5 days (*open circles*) and in the same cultures for 4 hr after the serum concentration was increased to 20% (*solid triangles*). *Bars* represent 90% confidence limits.

TABLE 1. *Rate of movement of individual cells in 2% and 20% serum (μm/hr)*

Serum concentration	Expt. 1	Expt. 2	Expt. 3	Average of three Expts.
20%	18.6 ± 6.6* (4)	33.3 ± 8.9 (6)	20.0 ± 8.3 (6)	25.4 ± 3.9 t.90† (16)
2%	7.8 ± 0.9 (4)	12.7 ± 5.1 (5)	7.9 ± 1.9 (3)	10.3 ± 2.5 t.90 (12)

* Data is given as mean ± standard deviation, with the number of cells measured in parentheses.
† Averages are given with ± 90% confidence limits.

The increase in the rate of movement of cells was measured from the paths of cells in the films taken during the first 24 hr after serum addition. The direction of cell movement appeared to be random, with a large variation in the rate of movement of individual cells. When several cells were followed, the average rate of cell movement in cultures with 20% serum was 2.5 times higher than that of cells grown in 2% serum (Table 1). The rate of cell movement increased soon after serum addition, since it was the same during the first 4 hr after serum addition as after 24 hr. In a separate experiment the rate of cell movement during the first hour after serum addition was compared to the rate during the next 3 hr and here also there was no difference (Table 2). The cells must begin to move more rapidly within minutes after serum addition. This immediate increase in the rate of movement and the much later increase in overlapping nuclei indicate that the increase in the rate of movement cannot be simply related to the release of contact inhibition of movement, as the latter was originally defined by overlapping cells (1).

Since serum addition both breaks cell contacts and increases cell movement, the increase in movement might be due to the serum effect on cell contacts, or the contacts could be broken by the stimulation of cell movement. If serum acted by reducing cell contacts, similar results should be obtained when cell contacts are reduced by lowering cell densities. In 2% serum, cells move more rapidly as cell density was decreased (Fig. 3). The maximum rate of movement at low densities in less serum was about equal to the rate of movement of cells stimulated by more serum at either high or low cell densities (*triangles*, Fig. 3). Similar results have recently been reported by Castor (12). These results suggest that cell movement is restrained by the adhesiveness of cell to cell contacts, and that increased serum concentrations act by weakening cell contacts, allowing cells to move as if they were at lower densities. The rate of movement of confluent cells appears to be a measure of the stability of cell contacts.

Correlation of cell movement with DNA synthesis

If the release of cell contacts and the concomitant increase in cell movement were closely related to the loss of growth inhibition, both movement and growth should be coordinately stimulated by various increases of serum concentration. To test this prediction, the increase in movement and the release of growth control were measured in cells grown to confluence in 0.5% serum and stimulated with various concentrations of serum up to 20%. Movement was measured by time-lapse cinematography during the first 4 hr after the addition of serum. The release of growth control was measured by autoradiography, as the increased percentage of cells incorporating thymidine into their nuclei during the first 20 hr after the increase in serum concentration. Both movement and DNA synthesis increased steadily to a maximum level as the serum was increased from 0.5 to 8% (Fig. 4). An increase in the serum concentration beyond 8% (to at least 20%) did not further stimulate either DNA synthesis or cell movement. Over the whole range of serum concentrations, the changes in movement and DNA synthesis are quantitatively parallel which suggests that the serum is acting on both through a common mechanism. Since the observations described above indicate that an increase in serum concentration breaks cell contacts that are restraining cell movement, and since cell contacts appear to be involved in growth control (2, 3), it is likely that the common mechanism is the release of cell contacts.

TABLE 2. *Rate of cell movement of individual cells in 8% serum for the first and the next 3 hr after serum addition*

Hours	Average	Number of cells measured	Range of values observed
0–1	21.2 ± 1.6*	19	13.5–32.0
2–4	21.8 ± 1.6	19	6.4–44.8

* Data given as μm/hr ± 90% confidence limits.

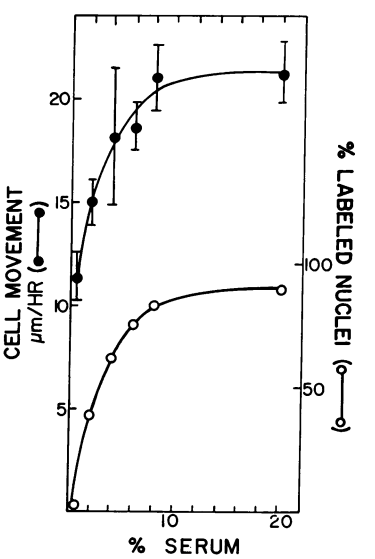

FIG. 4. Effect of added serum upon the average rate of cell movement for the first 4 hr after the increase (*closed circles*) and upon cells entering DNA synthesis during the first 20 hr after the increase (*open circles*). Cells were grown to confluence in 0.5% serum and the serum concentration was increased to the value plotted. The rates of movement are plotted with 90% confidence limits.

DISCUSSION

Increased serum concentrations appear to release contact inhibition of growth by reducing the adhesiveness of cells and allowing cell contacts to come apart. Our observations indicate that the cell contacts effective in limiting growth are not simply the casual touching of cells, but are stable adhesions strong enough to limit the movement of the cells. The nature of these stable cell contacts is unknown. They may be adhesions mediated by cell-surface glycoproteins (13) or may be closer cell associations, such as low-resistance junctions, that are dependent on cell adhesion (14).

The alteration by serum of the saturation densities attained by cell lines in culture has raised questions about the classical idea that cell contacts determine the saturation density (4, 8, 9). However, if because of a lower adhesiveness, cells in a high concentration of serum must reach a higher density before they can form stable cell contacts, the serum effect is consistent with the idea that contacts limit growth. Other apparently nonspecific factors, such as pH (15), that alter saturation densities could also act in this matter. This explanation removes the major reservation to the use of the original term "contact inhibition of growth" (16). Many other experiments also indicate that cell contacts and the cell surface are involved in the limitation of growth. Several proteases known to reduce the adhesion of cells also release contact inhibition (17). Cell lines that stop growth before confluence have not been found, although cell lines are easily produced with any saturation density above confluence (2). We suggest that cells that grow to low saturation densities are more adhesive than those that grow to higher saturation densities. Several different experimental approaches have shown an alteration of the cell surface associated with changes in saturation density; chemical groups that are "cryptic" on cells subject to growth control are exposed on the surface of cells showing little growth control (18–20). These cell-surface changes may be involved in cell adhesions.

This study has investigated the effect of serum upon two aspects of cell movement, the movement of cells over each other and the movement of confluent cells past each other. The difference in the time of increase in overlapping nuclei from the time of increase in the rate of movement suggests that these two serum activities depend on quantitatively or qualitatively different cellular responses. The movement of cells into a wounded area away from the monolayer is also stimulated by serum (9); this movement is required for cell division (5). The serum concentrations required for this movement appear to be much lower than those required to stimulate growth in a confluent monolayer (9) and, thus, this serum activity may also be different from the one that stimulates movement and growth in the intact monolayer. Elucidation of the relationships of these various serum activities requires further study.

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