SECTION IV. REPLICATE CULTURE METHODS

5. Single "Subculture" In Situ Using Glass Beads

G. M. Kolodny

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Massachusetts General Hospital
Boston, Massachusetts 02114

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Investigations of intracellular changes following release of contact inhibition have been conducted on cells released from contact inhibition by reseeding cells in a larger growth area after trypsinization, by addition of fresh serum (1,2), or by transforming these cells (3-6). These methods of releasing contact inhibition possess some disadvantages for the interpretation of experimental results. It is uncertain, for example, that early biochemical changes seen after reseeding trypsinized cells are due solely to loss of contact inhibition. Some may represent changes necessary to repair cellular damage caused by the trypsinization.

Serum stimulation in contact inhibited cells does not cause physical cell separation, and biochemical alterations seen after addition of fresh serum to a confluent culture might be due to stimuli other than those operating in an authentic release of contact inhibition. Similarly, virus infection of contact inhibited cells can and does activate processes other than those simply concerned with cell-cell contact.

A method of releasing contact inhibition without disturbing cell substrate attachment, or the chemical composition of the medium, should provide for an increase in the space available for growth of a confluent culture. Ideally, such an increase in growth area should be uniformly available to every cell within the culture. Increasing the growth area along a border of the culture leads to stimulation of cell division in only a very small and insignificant fraction of cells immediately adjacent to the border. Cells

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within the center of the culture are not released from contact inhibition (7).

In this laboratory we have studied the macromolecular changes accompanying release of contact inhibition by using a method by means of which a large fraction of cells in a confluent monolayer are released from contact inhibition of growth and division (8,9). This is accomplished without chemical treatment of the cells, without change of medium and in such a way that most cells are provided with free growth area. The procedure involves growing the cells to confluence on surfaces uniformly covered with glass beads 200 μ in diameter. When confluence has been attained, contact inhibition may be released by discarding the beads leaving behind numerous spaces, throughout the culture. Removal of the beads dislodges few if any of the cells. After release of contact inhibition by removing the beads, the cultures double in cell number following the first round of DNA replications, and continue to grow until they are again contact inhibited. Cell types used have included several cell lines and strains including both primary cell cultures of neonatal rat heart cells and established 3T3 and 3T6 mouse fibroblast cell lines.

Cell Cultures

Cells are cultured in Dulbecco's medium with 10% added calf serum, 75 units/ml penicillin, and 50 μ g/ml of streptomycin. Serum and medium are supplied by Grand Island Biological Co., Grand Island, N.Y. The cultures are incubated at 37°C in a humid atmosphere of 5% CO₂ in air. Cultures are checked periodically for contamination by microorganisms.

Maintenance of Cultures with Glass Beads

Cultures are seeded in the presence of glass beads. One gram of glass beads (200 μ avg. diam., 3M Company, Reflective Products

Division, St. Paul, Minn.), is placed in a 60 mm plastic tissue culture Petri dish (Falcon Plastics, Los Angeles, Calif.) after dry heat sterilization of the beads for 6-12 h at 170°C. The cells in 5 ml of medium are then seeded into the culture dish with the glass beads. By gentle tipping and swirling motions an even layer of beads is made to cover the bottom of the dish. The dish is carefully placed in the incubator and after a short time the cells settle between the beads. The cells attach on the surface of the dish between the beads and multiply until the spaces between the beads are completely occupied by adherent contact inhibited cells.

Medium in the Petri dish is changed every three days. The spent medium is removed with a curved-tip pasteur pipette fitted with a rubber bulb, slowly and carefully so as not to disturb the beads. Only about 85% of the spent medium is removed. If the dish is drained dry, some beads float off the surface when fresh medium is returned to the dish. The fresh medium is added slowly to the dish through a curved tip pipette lying against the side of the dish.

Removal of Beads

When the surface of the Petri dish between the glass beads is fully covered with cells, and at least 48 h after a change of medium, contact inhibition of cell division may be released by discarding the glass beads. The culture dish is tipped slightly to one side. The medium is sucked up into a wide bore pipette and then allowed to drip back on to the dish so as to wash all the glass beads to the low side of the tipped dish. The beads and medium are then pipetted together into a centrifuge tube which is spun at 1,600 g for one minute to pellet detached cells, dead cells and large debris with the beads. The cell-free supernatant medium is then returned to the tissue culture dish, which now contains a cell layer interspersed throughout with holes left by the discarded beads.

Release of Contact Inhibition

When the beads are discarded, the cells in the dish start to multiply and fill up the holes. Within 12-36 h, depending on the cell type, mitotic cells in large numbers can be seen within and at the periphery of the holes. In 72 to 96 h a confluent monolayer of contact inhibited cells has been formed.

Figure 1 shows a plot of DNA synthesis (thymidine incorporation) and mitotic index in the hours following release of contact inhibition by the glass bead method. There is a semisynchronous burst of DNA synthesis with a peak at about 30 hours after release followed by a semisynchronous peak in the mitotic index at about 40 hours.

Cell Growth

Normal cell growth characteristics are preserved in the presence of the beads and after their removal. Figure 2 is a plot of daily cell counts in replicate 3T3 cultures, before and after removal of beads. Media are changed typically 2 days after inoculating the cultures and every 3 days thereafter. The cell cultures grow logarithmically until confluent at about 10^5 cells/plate and remain contact inhibited until the beads are removed. After removal of the beads, the cells once again multiply logarithmically as shown in the figure. No change of media at times prior to removal of the beads is necessary for release of contact inhibition since growth curves similar to that seen here can be obtained without any change of medium throughout the period of growth with beads, removal of the beads, and reestablishment of a confluent culture at a higher cell density.

Comments

Certain considerations and precautions should influence use of the glass bead technique to maintain and release contact inhibi-

ition of cell division.

The beads must be sterilized with dry heat for at least 6 h at 170 °C. Sterilization in steam leads to caking of the beads so that they cannot be dispersed as a uniform layer. Sterilization for less than 6 h leads occasionally to growth of resistant microorganisms.

The use of beads smaller or larger than 200 μ has given less desirable behavior of the contact released monolayers. If the beads are smaller than 200 μ they do not settle well in the culture dish and do not provide sufficient space for growth between the beads. If the beads are larger than 200 μ there are many more cells between beads and a lower percentage is therefore released from contact inhibition when the beads are removed.

The presence of glass beads in intimate association with the cells has no apparent effect on their characteristic morphology, as determined by phase microscopy before and after removal of the beads. The glass beads used are composed of a soda-lime glass which does not provide a good substrate for growth of tissue culture cells. Therefore cells grown in the presence of the beads do not adhere to the beads and are apparently not damaged physically to any significant extent when the beads are removed.

Normally, beads are discarded at least 48 h after medium change and the same medium returned to the cultures to exclude the effect of releasing contact inhibition by medium change and fresh serum addition (7).

Mitotic indices, in our experience, are best obtained from mitotic cell counts using xl50 phase microscopy. The use of fixatives and stains in the counting of mitotic cells leads to an underestimation of mitotic cells. Mitotic cells round up and show decreased adhesiveness to the plastic substrate upon which they are growing (10). Agitation caused by adding fixatives and stains dislodges a great many of these. Observation of dislodged cells shown that greater than 50% are in mitosis.

FOOTNOTES

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LEGEND FOR FIGURES

FIGURE 1. DNA synthesis (thymidine incorporation) and mitoses in 3T3 cells following removal of beads. $2.5 \mu\text{C}$ H^3 -thymidine were added to each dish and incorporation during 20 min pulses measured as TCA precipitable cpm. These are plotted over the 42 h period following release of contact inhibition. Mitotic index in the same period is also plotted.

FIGURE 2. 3T3 cells were seeded on 60 mm plastic Petri dishes with 1 g of 200μ glass beads in each dish. Log counts of trypsinized cells before and after removing beads are plotted against days after seeding cultures. $\uparrow$ medium change. $\uparrow$ removal of beads.

FIGURE 1