

CONTACT-MEDIATED REVERSIBLE SUPPRESSION OF MYOGENESIS

II. Reversal of Suppression by Bromodeoxyuridine

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ABSTRACT

Chondrocytes from the vertebral columns of 11-day chick embryos were cultured in the continuous presence of 5-bromodeoxyuridine (BUdR). Under these conditions the cells form multilayers but synthesize little extracellular matrix as determined by toluidine blue metachromasia or sulfate-³⁵S incorporation into polysaccharide. Myogenic cells from the breast muscles of 11-day chick embryos formed myotubes when plated into BUdR-treated chondrocyte cultures. When plated on untreated chondrocyte multilayers or on multilayers which had been permitted to recover from BUdR treatment for 3 days, myogenic cells failed to form myotubes. Since extracellular matrix is present in untreated chondrocyte cultures and reappears in multilayers recovering from BUdR treatment, it is suggested that extracellular matrix is the active agent in the suppression of myogenesis. An attempt was made to duplicate the suppressing activity of multilayer cultures by using ion exchange resins as substrates for myogenic cells. Myotubes formed on acidic and basic resin particles. If extracellular matrix is the active suppressing agent, it may have to fulfill certain spatial distributional requirements before its activity is expressed.

INTRODUCTION

Skeletal muscle precursor cells (myogenic cells) will divide, migrate, and fuse to form multinucleated myotubes on a variety of substrates in vitro. Such substrates include glass, plastic, collagen, fibrin, paraffin, and cellulose acetate. When plated on nondividing multilayers of chondrocytes, liver cells, or muscle, however, myogenic cells attach to the multilayers but do not form myotubes (14, 15). Under these conditions, cell division and migration are suppressed, but the processes of fusion, per se, and the synthesis and assembly of myofibrillar proteins are not inhibited. Since mitosis may be required in order for a myogenic cell to acquire the capability to fuse (2, 14, 15), and since competent myogenic cells must contact each other before fusion can occur, the inhibitory effects of cellular mul-

tilayers on both mitosis and migration complement each other in preventing myotube formation. Although intimate contact between myogenic cells and the inhibitory multilayers is required for suppression of mitosis and migration, the mechanism operating during the interaction is not known. The experiments reported here suggest that extracellular matrix secreted by nondividing cellular multilayers may be the active suppressing agent.

MATERIALS AND METHODS

Cell Culture

Chondrocytes from the vertebral columns of 11-day chick embryos and myogenic cells from the breast muscles of similar embryos were prepared as described

by Nameroff and Holtzer (14). All cultures were initiated with 0.5×10^6 cells in 1.5 ml of medium (Eagle's minimal essential medium [Grand Island Biological Co., Grand Island, N. Y], 7.8 parts; horse serum, 1 part, 50% chick embryo extract, 1 part; Fungizone, 0.1 part; penicillin-streptomycin solution, 0.1 part) and were incubated in a water-saturated atmosphere containing 5% CO_2 in air at 37.5°C. Culture vessels were 35-mm Petri plates (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) containing 22-mm square cover slips coated with plasma clots (1 part chicken plasma; 1 part embryo extract). The medium was changed on the day after initiation of the culture and every other day thereafter.

Suppression of Extracellular

Matrix Synthesis

Chondrocytes were grown in medium containing 10 μg of 5-bromodeoxyuridine (BUdR, Calbiochem, Los Angeles, Calif.) per ml. Abbott and Holtzer (1) have shown that under similar conditions the cells synthesize little extracellular matrix. Control cultures had no additions to the medium. After 6 days the cultures were split into three groups. Group 1 consisted of BUdR-treated cultures which were washed

twice with balanced salt solution (BSS) and incubated for the next four days with normal medium (i.e. without BUdR). Group 2 consisted of BUdR-treated cultures washed twice with BSS and then incubated with medium containing 10 μg of BUdR per ml (i.e. they were continued in culture in the presence of BUdR). Group 3 consisted of the control cultures which were washed with BSS and incubated in normal medium. On successive days, starting on day 6, several cultures of each group were exposed to sulfate- ^{35}S ($\text{Na}_2\text{S}^{35}\text{O}_4$, 738 mCi per mmole, New England Nuclear Corp., Boston, Mass.) at a concentration of 5 μCi per ml for 24 hr. Incorporation was stopped by removing the medium and freezing the cells by dipping the culture dish bottom into a mixture of dry ice and acetone. The cells were stored frozen until the end of the experiment when incorporation of the isotope into polysaccharide was assayed according to the method of Nameroff and Holtzer (13). In brief, the method consisted of digesting the cells with pronase, precipitating with cold trichloroacetic acid (TCA), and dialyzing the supernatant against sodium sulfate solutions and finally against water. Samples were taken for counting in a liquid scintillation counter and correction was made for ^{35}S decay. Sulfated polysaccharides were characterized by electrophoresis on cellulose

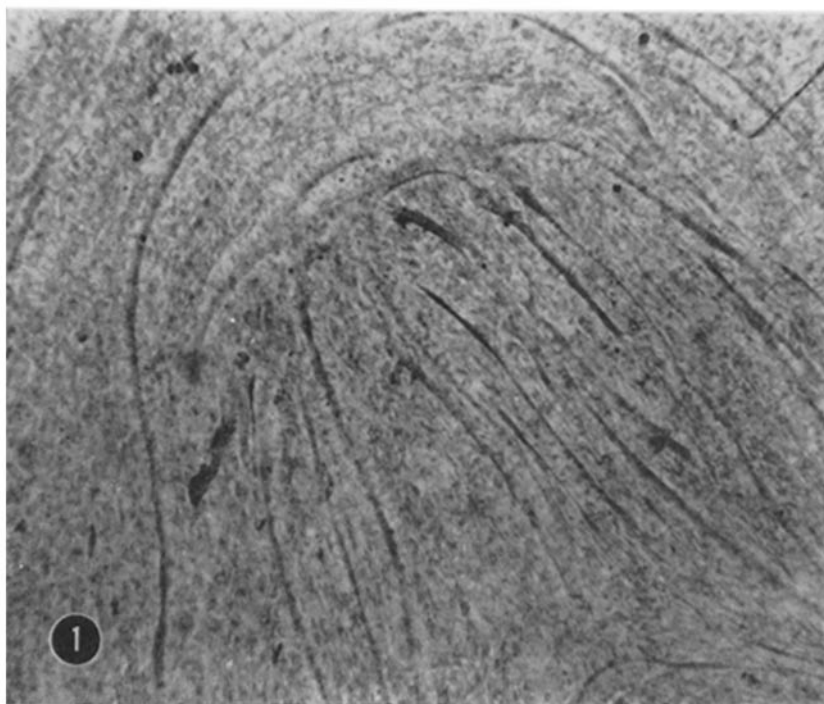


FIGURE 1 Myotubes which formed on a BUdR-treated chondrocyte multilayer. Most myotubes were not as large as those depicted here. $\times 100$.

acetate strips in pyridinium formate buffer (0.1 M, pH 3.0, 150 v, 80 min, 25°C). The strips were air-dried, cut into 1.0-cm sections, and counted in the scintillation counter. Standards for migration comparison were chondroitin sulfate C (Calbiochem) and hyaluronic acid (Mann Research Labs. Inc., New York).

Several cultures from each group were fixed and stained for acidic polysaccharide at various times. The cultures were rinsed twice with BSS, fixed for 5 min in absolute methanol, and stained for 5 min in 0.1% toluidine blue in 30% methanol in water. Cover slips were then dipped for a few seconds into acetone, acetone-xylene (1:1), and cleared in xylene before mounting (6).

Effect of Matrix Suppression on Myogenesis

Myogenic cells were plated into several cultures from each chondrocyte group on successive days after removal of BUdR from group 1. In some cultures, nonradioactive thymidine (Calbiochem), at a concentration of 0.2 mM, was added to overcome any effect of residual BUdR. Cultures were fixed after 3 days and stained as described above.

DNA Synthesis

DNA synthesis in BUdR-treated and control chondrocyte cultures was assayed as incorporation of thymidine-methyl-³H into TCA-precipitable material. Because of the competition between thymidine and BUdR for uptake into DNA, and because of the uncertainty in eliminating trace quantities of BUdR by washing, the following procedure was adopted. Untreated controls were incubated in BUdR (10 µg per ml) for 30 min. Both BUdR-treated cultures

and the preincubated controls were then washed three times with BSS and incubated in medium containing 1.0 µCi of thymidine-methyl-³H per ml for 2 hr. The cultures were then exposed for 30 min to medium containing 0.2 mM unlabeled thymidine. After a wash with BSS, the cells were placed in 0.25% trypsin until they detached from their substrates. Trypsin was removed by centrifuging the cells and washing once with BSS. The cells were subjected to one cycle of freezing and thawing and were homogenized in glass tissue grinders at 0°C. TCA was added to 10%, and insoluble material was collected by suction onto glass fiber filter pads (Whatman GF/B). The pads were washed with alcohol-ether and alcohol and were then air-dried and counted in 10 ml of scintillation fluid (3).

Cell-Resin Interactions

For experiments in which attempts were made to mimic extracellular matrix, the following anion and cation exchange resins were obtained from Fisher Scientific Co., Fair Lawn, N. J.: (a) Rexyn 201 (Cl), polystyrene quaternary amine, bead form, 200–400 mesh particles, ionic form R₄N⁺, Cl⁻, strong base, (b) Rexyn 203 (OH), polystyrene amine, bead form, 16–50 mesh particles, ionic form R₄NH⁺, OH⁻ and RNH₂⁺, OH⁻, weak base, (c) Rexyn 102 (H), methacrylic acid containing carboxyl groups, bead form, 100–200 mesh particles, ionic form RCOO⁻, H⁺, weak acid; (d) Rexyn 101 (H), sulfonated polystyrene copolymer, bead form, 40–100 mesh particles, ionic form RSO₃⁻, H⁺, strong acid. The resin particles were sterilized in 10% formaldehyde, washed in sterile water, and finally in the saline base of the tissue culture medium (Earle's BSS). Myogenic cells were plated into 35-mm culture dishes each contain-

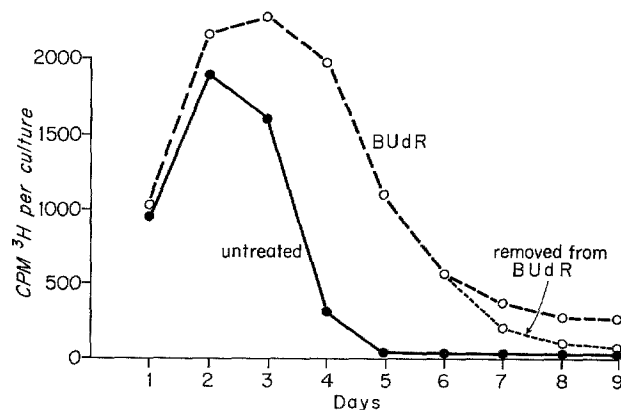


FIGURE 2 Incorporation of labeled thymidine into TCA-precipitable material. Ordinate represents cpm per culture per 2 hr of exposure to the isotope. BUdR-treated cells divided for a longer period of time than controls. When multilayering occurred, BUdR-treated cells appeared to continue to turn over at a higher rate than controls. Removal of BUdR led to a reduction in the rate of thymidine incorporation.

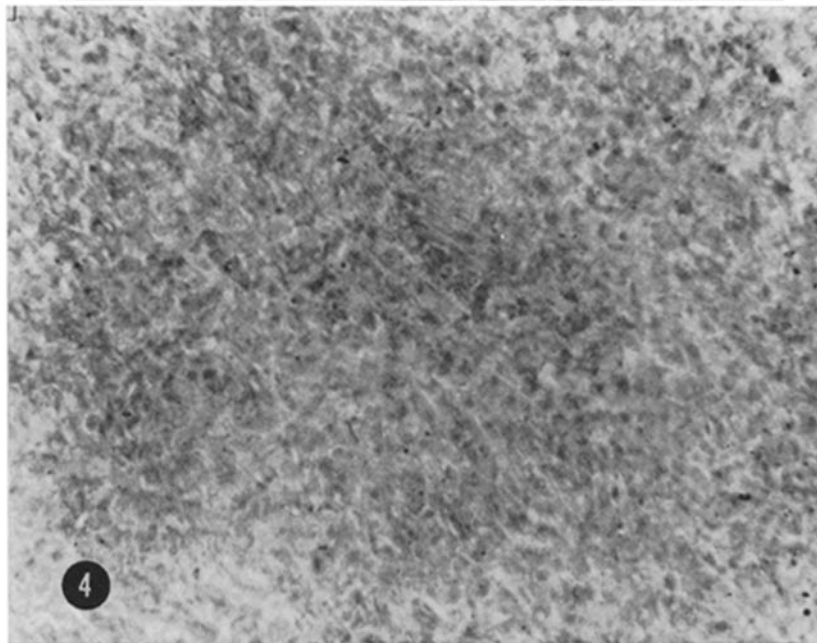
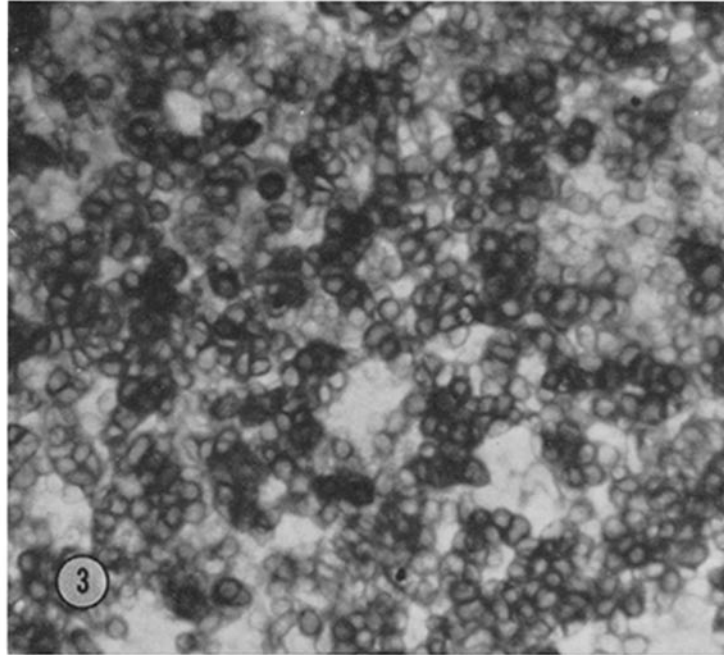


FIGURE 3 Control chondrocyte culture, 10 days old, stained with toluidine blue. Capsules are apparent around most of the cells. Matrix is intensely purple. $\times 200$

FIGURE 4 Sample of the same cells used for Fig. 3 but grown in the continuous presence of BUdR. No metachromatic matrix is seen with toluidine blue. $\times 200$.

ing a "monolayer" of resin beads in medium. After an overnight incubation, the resin particles with attached cells were removed from the original dishes and dispensed at a much lower density into new dishes with medium. In the low density platings the resin particles could cover $\frac{1}{8}$ - $\frac{1}{4}$ of the surface of a 35-mm dish if allowed to roll together to "confluence." Myogenic cells were also plated directly on resin particles set up initially at low density. In this type of culture the cells could attach either to the resin beads or to the surface of the culture dish. At various times after initiating the cultures, the resin particles were washed once with BSS and were fixed with alcohol-formalin-acetic acid (20:2:1). They were then handled as tissue with regard to staining, dehydrating, clearing, and mounting. Cultures initially set up with resin beads at low density were photographed with phase contrast optics in the living state. Myogenic cells were also cultured under standard conditions with 1-10 mg of chondroitin sulfate per ml of medium.

RESULTS

Suppression of Extracellular Matrix Synthesis

If extracellular matrix were the active agent in suppression, then cells which were not making matrix should permit myogenic cells to divide, migrate, and form myotubes. Chondrocytes can be kept from synthesizing matrix by culturing them in the presence of BUdR, and, at appropriate concentrations, BUdR will inhibit chondroitin sulfate synthesis but permit cell division (1, 4, 5, 18). Chondrocytes were therefore grown for 6 days in the presence of BUdR at a concentration of 10 μ g per ml of medium. On the sixth day, BUdR was removed from the medium. Myogenic cells were inoculated into several such cultures on day 6. Each day thereafter, myogenic cells were plated into cultures which had been free of BUdR for an additional 24 hr. Myogenic cells were also plated onto untreated chondrocyte substrates and onto clot-coated cover slips as controls. All cultures were fixed and stained 3 days after plating the myogenic cells.

Myotubes formed in cultures which had been treated with BUdR for 6 days and which received an inoculum of myogenic cells on day 6 immediately after the BUdR was removed (Fig. 1). Myotubes also formed in BUdR-treated cultures when the myogenic cells were plated into them 1 day after removal of BUdR. When myogenic cells were plated on treated cultures 3 or more days after re-

moval of BUdR, no myotubes formed. No myotubes were observed on untreated chondrocyte substrates. Most of the myotubes which formed on BUdR-treated substrates were smaller and shorter than those made by samples of the same cell suspension when plated on plasma clots.

Under phase optics, the living BUdR-treated chondrocytes were flattened and stellate in appearance. They multiplied as fast or faster than controls (Fig. 2), and both treated and untreated cells formed multilayers. Staining with toluidine blue showed that untreated chondrocytes deposited large amounts of metachromatic matrix (Fig. 3), while BUdR-treated cultures displayed little or no metachromatic material (Fig. 4). 3 days after removal of BUdR, diffuse metachromasia appeared in the treated cultures. An independent assay of matrix synthesis, incorporation of sulfate- 35 S into acidic polysaccharide, confirmed the histological findings with toluidine blue. As shown in Fig. 5, sulfate- 35 S incorporation was markedly reduced in cultures exposed continuously to BUdR. After removal of BUdR, matrix synthesis remained at a

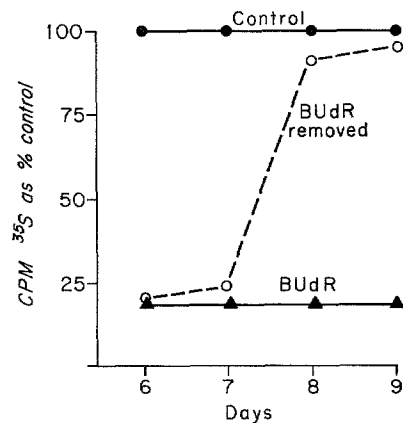


FIGURE 5 Incorporation of sulfate- 35 S into acidic polysaccharide in untreated (control), BUdR-treated (BUdR), and BUdR-treated cells removed from the analogue on day 6 (BUdR removed). Ordinate represents incorporation per culture (average of six cultures) expressed as per cent of control. Exposure to the isotope was for 24 hr. Controls incorporated between 1.0×10^5 cpm and 1.5×10^5 cpm. Although incorporation in cultures removed from BUdR approaches control values, there are probably more cells in the recovering cultures than in controls (see Fig. 2). Therefore, the rate of incorporation per cell is lower than in controls. Sulfated polysaccharides isolated from all cultures had identical electrophoretic mobilities and migrated like authentic chondroitin sulfate.

low level for 1-2 days and then rose to a value comparable to that of untreated controls. Thus, myotubes formed in cultures which had no metachromatic matrix, but when matrix reappeared histologically and chemically, myotube formation was suppressed.

Cell-Resin Interaction

If extracellular matrix is the agent which suppresses mitosis and migration of myogenic cells, then some component of matrix might duplicate the effect if presented to myogenic cells in an appropriate way. The major macromolecular components of cartilage matrix are collagen and a proteoglycan consisting of a polypeptide chain to which the polysaccharide, chondroitin sulfate, is

linked. Collagen, when used as a substrate, is known to support mitosis and movement of myogenic cell as well as subsequent fusion to form myotubes (10). It was therefore decided to test the possibility that chondroitin sulfate might inhibit mitosis and movement. Myogenic cells were cultured in medium containing 1-10 mg of chondroitin sulfate per ml. Mitosis, migration, and myotube formation occurred in all cultures at about the same rate. At no time were cultures in any concentration of chondroitin sulfate distinguishable from cultures with no added polysaccharide. The conclusion drawn from this experiment was that chondroitin sulfate *in solution* did not inhibit mitosis and movement. The experiment did not rule out that chondroitin sulfate which was

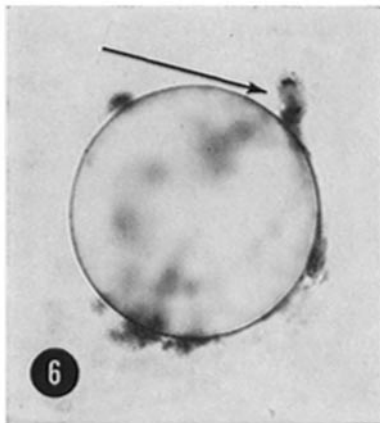


FIGURE 6 A cell caught in mitosis on a weak acid resin bead. $\times 400$.

FIGURE 7 Myotubes wrapping around a weak base resin particle. Much of the bead is out of the focal plane, $\times 200$.



fixed in space, as in authentic matrix, might be active. Since chondroitin sulfate is a polyanion, if it were the active component of matrix, it would present negatively charged groups to myogenic cells. Myogenic cells were therefore cultured on cation exchange resin beads (particles with negative charges fixed in space) in an attempt to mimic the effect of cellular substrates. The cells were also cultured on anion exchange resins (particles with fixed positive charges) as controls consisting of organic polymers similar to those in the acidic (cation) resins but of different charge. Cells were cultured under two different conditions with each type of resin. In one type of experiment, myogenic cells were permitted to attach to confluent resin beds in 35-mm dishes for 12–16 hr. The resin beds were then diluted into other dishes by dispensing the particles from a single bed into four to eight plates. In a second experiment, the cells were inoculated directly into dishes containing dilutely plated resin beads. Under these conditions cells could attach either to the beads or to the bottom of the dish. Mitotic figures (Fig. 6) and myotubes (Fig. 7) were observed on all types of resin particles. Myogenic cells also divided and formed myotubes on the surfaces of the culture dishes containing dilutely plated resin beads. In cultures containing acidic resins, the indicator (phenol red) in the medium was bright yellow in the incubator environment (5% CO₂ in air). With basic resins, the medium was first deep red but, particularly with the strong base, soon became clear, probably as a result of absorption of the indicator by the resin. No attempt was made to measure the pH of the medium. Clearly, the fixed charges of the ion exchange beads did not inhibit mitosis and myotube formation either on the surfaces of the beads or in the “bulk phase” of the culture medium, and therefore did not mimic the effect of cellular multilayers.

DISCUSSION

Although the experiments reported here are consistent with the hypothesis that extracellular matrix is the active agent in suppressing mitosis and movement of myogenic cells, they do not conclusively prove that hypothesis. Previous experiments have ruled out a diffusible suppressor (14). In the present experiments, BUdR was used to prevent matrix synthesis by chondrocytes. There was a lag of 1–2 days after release from BUdR before chondrocytes resumed matrix synthesis at a high rate. It is not known whether all chondrocytes recovered from the treatment. Since BUdR-treated chondrocytes

divided during the lag period, it is inferred that myogenic cells could divide and move while the chondrocytes recovered. Experimental testing of this inference is in progress. The observation that the myotubes which formed in treated cultures were smaller than controls can be explained if there were a “race” between accumulation of matrix by recovering chondrocytes and mitosis and movement of myogenic cells. Either fewer contacts between competent myogenic cells occurred as matrix appeared or fewer mitoses were permitted (or both). After matrix synthesis reached a high rate and was detectable as metachromatic material accumulating in the cultures, mitosis and movement were no longer permitted.

Cell-cell contacts undoubtedly occurred between myogenic cells and BUdR-treated chondrocytes. If cell-cell rather than cell-matrix contacts are required for suppression, then not all such contacts can mediate the inhibition of mitosis and movement. BUdR-treated cells might not make or transmit hypothetical suppressors even though contacts occur. Alternatively, the kinds of contacts which BUdR-treated cells are capable of making may be different from those which untreated cells make. It has been suggested that the surfaces of BUdR-treated chondrocytes are different from those of normal cells (1). When labeled myogenic cells were plated into untreated chondrocyte cultures, most of the labeled cells appeared to be surrounded by extracellular matrix (15). These observations were carried out with the light microscope. It is possible that there were thin processes, not resolvable by light microscopy, by which myogenic cells contacted other cells in the cultures. This possibility is being investigated with electron microscopy. If there are such cell-cell contacts, suppressing information might be transmitted from chondrocytes directly to myogenic cells (19). Matrix contacts, as causes of suppression, could not be ruled out, however, even if cell-cell links were found, since the cell surface would still be largely in apposition to extracellular material.

A direct test of the hypothesis that matrix is the suppressing agent would require cell-free intact matrix. So far, it has not been possible to obtain such material. Attempts have therefore been made to construct matrix models and test their ability to mimic the suppressing activity of cellular multilayers. One such attempt is reported here. Myogenic cells were cultured on ion exchange resin particles with fixed charges. Mitosis and myotube formation occurred on such resins. Both acidic and basic resins permitted myogenesis on their surfaces.

If the fixed negative charges of matrix are involved in the suppressive interaction, then either the density or spatial distribution of the resin charges was sufficiently different from that of matrix to permit mitosis and myotube formation. It is possible, for example, that myogenic cells must "see" charges all around their surfaces in order to be suppressed. This speculation is tempting in light of the observation that most myogenic cells are apparently completely surrounded by matrix in suppressing cultures (15). It is also possible, especially since neither acidic nor basic resins suppressed myogenesis, that the charge on the material surrounding a myogenic cell has nothing to do with suppression. Totally surrounding a cell by any type of matrix may be sufficient to inhibit its mitotic activity and movement. According to this notion, a myogenic cell could divide and move in any (nontoxic) asymmetric environment, while a symmetric environment would suppress these activities. In recent experiments (Leung and Nameroff, in preparation) myogenic cells were suspended in plasma clots. They formed few myotubes. The cells could be recovered from the clots and replated on cover slips where they then divided and formed muscle. Control cells on top of clots or under clots (asymmetric environments) divided and fused to form myotubes. Therefore, if the extracellular matrix inhibits mitosis and movement, the distribution of the material rather than its specific composition may be the determining factor in its activity.

The proposition that extracellular materials play an important role in the regulation of cell differentiation is not new. Weiss (21) and Moscona (11) have suggested this possibility, and more recent work (7, 8, 20) has further implicated matrix constituents as participants in the control of cell behavior. Suppression of specific cellular activities must be an important process in embryogenesis. Organs and tissues attain certain sizes and shapes in part because growth processes cease. In extreme cases, cells die in order to create specific structures (9, 17). Attention has largely been paid, however, to the dramatic inductive events in embryogenesis in which new cell types or new organ anlagen appear where none existed before. Yet, even these interactions may be partly suppressive in character if they disfavor activities of locally unwanted cells. Further study of this and other suppressive interactions (4, 12) may permit a more comprehensive understanding of mechanisms operating in cellular differentiation.

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