GROWTH OF MAMMALIAN CELLS ON SUBSTRATES COATED WITH CELLULAR MICROEXUDATES

I. Effect on Cell Growth at Low Population Densities

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ABSTRACT

Mammalian and avian cells cultured on glass or plastic substrates produce microexudates of cellular macromolecules which remain bound to the substrate when the cells are detached. The gross macromolecular composition of microexudates from a range of diploid, heteroploid, and virus-transformed cells was determined with cells labeled with radioisotopes. Significant differences in the amounts of cellular glycoproteins, proteins, and RNA present in microexudates were found between different cell types and between cells of the same type at different stages of growth. Inoculation of cells onto substrates "coated" with microexudates altered their growth behavior. Microexudates from exponentially growing subconfluent homotypic and heterotypic cell populations enhanced the growth of mouse and chick embryo cells seeded at very low densities, but similar microexudates had no effect on the proliferation of cells seeded at higher densities. The enhanced growth of low-density cell populations seeded on microexudates was compared with the growth enhancement produced by feeder cell layers and conditioned medium.

Mammalian cells cultured in vitro on solid substrates produce a thin layer of "microexudate" that remains attached to the substrate when the cells are removed. This material is not detected by light microscopy, but can be demonstrated by electron microscopy (1, 3, 5, 14, 27, 31, 37, 40, 45), ellipsometry (25, 32, 34), mixed hemadsorption (26, 42), immunofluorescence techniques (3, 26, 43), and the incorporation of radioisotopes (8, 34, 44, 45). The microexudates deposited on substrates by mammalian cells are actively synthesized by the cell and do not result merely from the passive leakage of intracellular macromolecules onto the substrate (18, 25). The demonstration of cell-specific antigens (3, 26, 42, 43), virus receptors (25), and lectin-binding sites (23, 25) in cellular microexudates, together with data on the synthesis (25) and chemical characterization of such materials (8), have prompted the proposal that microexudates represent cell surface macromolecules derived from the so-called "cell coat" (25).

In this paper we present information on the composition of cellular microexudates deposited on glass and plastic substrates by a variety of diploid, heteroploid, and transformed cells and on alterations in the growth behavior of cells plated at

low densities onto substrates "coated" with cellular microexudates.

MATERIALS AND METHODS

Cells

Chick embryo (CE) cell cultures were prepared by trypsinization of decapitated, eviscerated 10-day old chick embryos, as described previously (29). After trypsinization, 6×10^6 cells were seeded in 10 ml of culture medium into 75-cm² plastic flasks (Falcon Plastics, Division of Bioquest, Oxnard, Calif.) to initiate primary cultures, which were subcultured after 4–6 days to provide the secondary cultures used in experiments. Primary and secondary mouse embryo (ME) cell cultures were prepared by the same procedure from 15–16-day old CBA mouse embryos from single litters. Both CE and ME cell cultures were grown in Eagle's basal medium supplemented with 10% calf serum. Feeder layers of CE or ME cells for growth enhancement experiments were produced by seeding 2×10^5 X-irradiated cells/50-mm dish. Feeder cells were irradiated with 5,000R at approximately 1,000R/min at a distance of 10 cm. No detectable growth of irradiated feeder layer populations occurred in the 6-day experimental period. Balb/C mouse 3T3 cells and similar 3T3 cells transformed by simian virus 40 (SV3T3) were cultured in Eagle's basal medium supplemented with 10% calf serum as described previously (24). Two variant cell lines (R-SV3T3-10 and R-SV3T3-14) showing partial reversion of their transformed properties were isolated from the SV3T3 cell population on the basis of their increased resistance to agglutination by concanavalin A by the methods described by Culp and Black (9), and cultured under the same conditions as the parent SV3T3 cells. Human diploid lung (W138) and skin (AL) fibroblasts were obtained from Dr. T. Shows and cultured in Eagle's basal medium plus 10% calf serum. Rat liver cells (RLB) were obtained from Dr. C. Borek and cultured in F12 medium supplemented with 10% fetal calf serum (4). African green monkey kidney cells were grown in Eagle's basal medium supplemented with 10% calf serum. Culture media and sera were obtained from the Grand Island Biological Co., Grand Island, N. Y. Unless stated otherwise, cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air in 50-mm plastic Petri dish cultures (20 cm² surface area) (Falcon Plastics) or 75 cm² plastic flasks (Falcon Plastics).

Conditioned Medium (CM)

Active CM preparations were obtained from 4–5-day secondary CE cultures and mouse L929 cells maintained in 2-liter glass roller flasks, as described elsewhere (24).

Cell Counts

The culture medium was aspirated from cell cultures in 50-mm plastic dishes and the cells were washed briefly with 5 ml of phosphate-buffered saline (PBS). The PBS was then aspirated and replaced by 1.0 ml of a prewarmed solution of 0.2% EDTA in calcium- and magnesium-free saline (CMF-BSS) or 0.2% EDTA and 0.2% trypsin in CMF-BSS. Dishes were incubated at 37°C for 20 min in the case of EDTA alone, or for 10 min for EDTA and trypsin, after which 2.0 ml of PBS was added, and the final suspension of detached cells was prepared by repeated pipetting. The number of cells in the suspension was then counted either by direct counts in a microscope hemocytometer chamber or by a Coulter electronic particle counter (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.) calibrated to provide equivalent counts to the mean of six hemacytometer counts of the same cell type. Each experimental value shown in the figures in the Results section represents a mean from cell counts on at least three separate dishes.

Radiolabeling and Assay of Substrate-Bound Cell Macromolecules in Microexudates

The macromolecular composition of cellular microexudates bound to glass or plastic substrates was assayed by labeling cells *in situ* with appropriate radioisotopes, followed by the detachment of cells from the substrate and measurement of the amount of radioactively labeled material remaining bound to the substrate devoid of cells. Cells were grown in normal culture medium in either 50-mm plastic dishes or 75-cm² plastic flasks. To assay substrate-bound cellular macromolecules deposited by subconfluent cultures, the culture medium was removed from cells 24 h after inoculation, when the culture was approximately 30% confluent, and replaced with fresh medium containing either 2.5 μCi/ml [³H]glucosamine or 1 μCi/ml [³H]uridine to label "glycoproteins" and RNA, respectively. For the labeling of proteins, cell cultures at a similar stage of confluency were incubated in leucine-deficient medium supplemented with 5.0 mg/liter leucine and 1 μCi/ml [³H]leucine. The subconfluent cells were incubated in the presence of the various radioisotopes for 48–56 h until the cultures were 50–60% confluent. The cells were then detached from the substrate by 0.2% EDTA in CMF-BSS and the amount of radioactivity associated with cellular material bound to the substrate was measured as described below. To identify substrate-bound macromolecules in microexudates deposited by confluent cell cultures, medium supplemented with radioisotopes at the same concentrations used for subconfluent cultures was added when cultures had just become confluent. After further incubation for 48 h the cells were detached from the surface of the culture vessel as described above, and the incorporation of radioactivity into cellular macromolecules remaining on the substrate was measured by the following method. The culture medium was first aspirated from cells attached to the substrate, the attached cells were washed twice with PBS, and then the cells were detached

by incubation with 0.2% EDTA in CMF-BSS for 20 min at 37°C. A 0.1-ml aliquot of the supernate (detached cells in EDTA solution) was removed and precipitated by 10% trichloracetic acid (TCA) after addition of 100 μg of carrier bovine serum albumin (BSA) to determine the total amount of "cell-associated" radioactivity. The substrate devoid of cells was then washed three times with distilled water and an aqueous solution of 1% sodium dodecyl sulfate (SDS) added. The culture vessel was then shaken for 30 min at 37°C and an aliquot of the SDS-extract assayed for TCA-precipitable radioactivity as described above to measure the amount of labeled substrate-bound macromolecules. The latter value was expressed as a percentage of the total cell-associated radioactivity. Measurements of incorporated radioactivity in the various samples were made in Aquasol scintillation fluid (New England Nuclear, Boston, Mass.) with a Beckman LS-230 liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Cell Growth on Microexudates from Homotypic and Heterotypic Cells

To study the growth behavior of cells plated onto substrates coated with microexudates from cells of the same (homotypic) or a different type (heterotypic), microexudate donor cells were seeded at the various densities given in the Results section in 50-mm plastic dishes and then detached from the dish at intervals when the required cell population density had been achieved. Donor cell populations were detached with 0.2% EDTA in CMF-BSS and the number of cells counted as described above. The dishes, now devoid of cells, were washed three times with PBS, and new cells of the same or a different type were inoculated onto the surface of the dishes and their growth was compared with that of control cells seeded at identical densities onto untreated plastic dishes. Secondary ME or CE cell cultures were used as donor cultures for substrate-bound microexudates in most experiments. For confluent donor cultures, 3×10^6 ME or CE cells were added to 50-mm plastic dishes (1.5×10^5 cell/cm^2). For sparse subconfluent cultures, 1×10^5 CE or ME cells were inoculated per dish (5×10^3 cells/cm^2). 5 ml of culture medium was used per dish for both sparse and confluent cultures, giving a ratio of medium volume to available cell-growth area of 0.3 ml/cm^2. Donor ME or CE cell cultures seeded at the high density were confluent 24 h later, whereas the sparse cultures did not become confluent within the 6-day experimental period.

Radioisotopes

L-[4,5-^{3}H]Leucine, sp act 35 Ci/mmol and [5-^{3}H]uridine 5-T, sp act 20.4 Ci/mmol, were obtained from New England Nuclear, and [1-^{3}H]glucosamine, sp act 2.4 Ci/mmol was from Amersham-Searle Corp., Arlington Heights, Ill.

Reagents

Thrice-crystalline trypsin was purchased from the Worthington Biochemical Corp., Freehold, N. J.; EDTA from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y., SDS from MC&B Manufacturing Chemists, Norwood, Ohio; and BSA from Miles Laboratories, Inc., Kankakee, Ill.

RESULTS

Demonstration of Substrate-Bound Radioactively Labeled Cellular Macromolecules

The respective incorporations of [^{3}H]glucosamine, [^{3}H]leucine, and [^{3}H]uridine into cellular glycoproteins, proteins, and RNA-containing moieties deposited on the surfaces of plastic dishes by a variety of diploid, heteroploid, and virus-transformed and revertant-transformed cell lines are summarized in Table I.

The results indicate that a significant amount of radioactively labeled cell-associated proteins and glycoproteins remain bound to the surface of the dish after detachment of the cells. As reported previously (22, 25, 39), the detachment of cells from plastic surfaces is not accompanied by any reduction in cell viability. Similarly, dye-exclusion viability measurements on cells used in the present experiments demonstrated that the EDTA detachment procedure did not cause any reduction in cell viability. Although it is recognized that cell damage would result in the release of proteinaceous material onto the substrate, previous studies on the nature of cellular microexudates have examined this problem in detail and have established that microexudates contain functional cell surface components (42–44) that are actively synthesized (18, 25), rather than released passively by damaged cells. Microscopic examination of culture dishes after cell detachment revealed no visible cells on the substrate, a finding in agreement with previous studies using accurate thin-film optical analysis (26). Furthermore, no cell colonies could be recovered from dishes after 4 wk of incubation.

The results in Table I indicate a significant variation in the amount of "glycoproteins" deposited by untransformed cells and their transformed derivatives, though the revertant transformed cell lines, R-SV3T3-10 and R-SV3T3-14, more closely resemble the original parent 3T3 cells. Cultivation of cells from the same population at equivalent densities on glass and plastic substrates revealed

that the overall composition of radioactively labeled macromolecules deposited on the different substrates did not differ significantly (Table II). A more detailed characterization of the cellular macromolecules found in microexudates deposited on different substrates, together with data on the release of similar macromolecules into the culture medium, will be presented elsewhere (G. Poste and L. Weiss, manuscript in preparation).

Growth Behavior of Cells Inoculated onto Substrates Coated with Cellular Microexudates

In general, the growth rate of mammalian cells in culture varies directly with the initial cell concentration. Below certain minimum cell concentrations, little or no net growth occurs (12, 16, 30, 34). This phenomenon is illustrated by the growth curves for secondary CE or ME cell cultures seeded at different densities in 50-mm untreated plastic dishes (Fig. 1), when cells seeded at densities of less than 5×10^4 cells/dish (2.5×10^3 cells/cm^2) show no net growth.

Recent studies by Yaoi and Kanaseki (45) have shown that the growth of CE cells inoculated at similar low densities was enhanced by seeding cells onto glass substrates on which CE cells had been previously growing and which were coated with what these authors described as a "carpet" of cellular material. To determine the relationship between this carpet of cellular macromolecules and

TABLE I

Macromolecular Composition of Mammalian Cell Microexudates Deposited on Plastic Substrates

Cell	Density	Radiolabeled substrate-bound macromolecules as % total cell-associated radiolabeled material		
		Glycoprotein	Protein	RNA
	cells $\times 10^4/cm^2$			
CE	5	9.3 ± 2.5	4.4 ± 1.1	0.4 ± 0.09
	22	14.8 ± 5.7	6.9 ± 2.3	0.2 ± 0.05
ME	5	11.3 ± 2.4	5.7 ± 1.7	0.2 ± 0.06
	21	17.2 ± 4.7	6.2 ± 2.6	ND
3T3	4	14.3 ± 3.2	7.3 ± 3.3	0.5 ± 0.08
	9	18.5 ± 5.5	6.3 ± 1.9	0.2 ± 0.04
SV3T3	11	3.9 ± 0.6	2.5 ± 0.9	0.1 ± 0.05
	26	2.2 ± 0.8	1.9 ± 0.6	0.1 ± 0.03
R-SV3T3-10	9	16.2 ± 3.9	5.8 ± 1.3	0.3 ± 0.09
R-SV3T3-14	11.5	15.6 ± 4.6	5.1 ± 1.8	0.2 ± 0.07

Mean values ± SE derived from five separate experiments. 60 50-mm plastic dishes were used for the assay of glycoprotein, protein, and RNA in each experiment.
ND = not done.

TABLE II

*Macromolecular Composition of Mammalian Cell Microexudates Deposited on Glass and Plastic Substrates**

Cell	Density	Substrate	Radiolabeled substrate-bound macromolecules as % total cell-associated radiolabeled material		
			Glycoprotein	Protein	RNA
	cells $\times 10^4/cm^2$				
CE	22	Glass	15.3 ± 4.6	5.1 ± 2.4	0.19 ± 0.03
	23	Plastic	15.8 ± 5.3	5.4 ± 1.4	0.24 ± 0.07
3T3	9	Glass	18.6 ± 6.3	6.2 ± 2.6	0.21 ± 0.03
	9	Plastic	19.3 ± 5.5	6.4 ± 1.7	0.16 ± 0.04
SV3T3	27	Glass	3.14 ± 1.4	1.82 ± 1.1	0.17 ± 0.06
	26	Plastic	2.45 ± 0.9	1.98 ± 0.7	0.12 ± 0.06

* Mean values ± SE derived from three separate experiments. 60 50-mm plastic dish cultures were used for the assay of glycoprotein, protein, and RNA in each experiment.

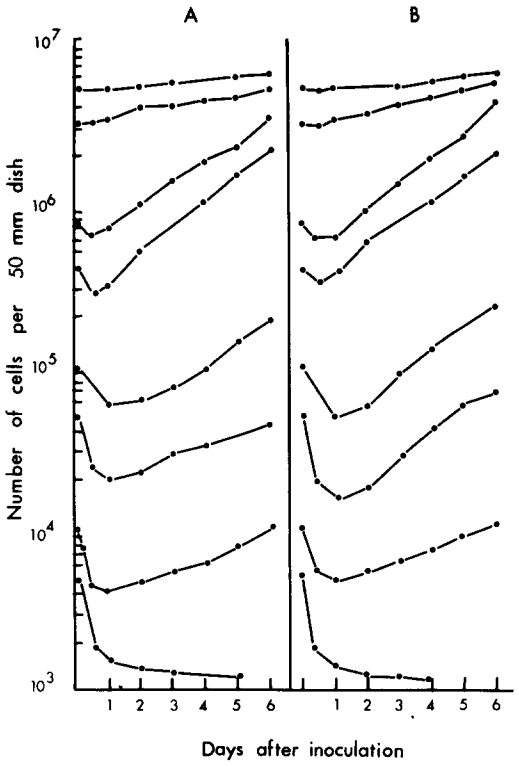

FIGURE 1 Growth of chick embryo (A) and mouse embryo cells (B) seeded at different densities. Each point represents a mean value from cell counts on four separate 50-mm dishes.

the microexudates detected in our own system, the growth behavior of low density CE and ME cell populations seeded onto the surface of plastic dishes coated with microexudates derived from cells of the same type (homotypic) was investigated and their growth was compared with that of cells from the same population at similar densities on untreated plastic dishes. The results indicate that the growth of both CE (Fig. 2 A) and ME cells (Fig. 2 B) seeded at low densities on homotypic microexudates is significantly enhanced compared with the control cells on untreated substrates. However, the growth rate of CE or ME cells seeded at high densities is not significantly altered by the presence of a microexudate on the substrate (Fig. 2 A and B).

Treatment of the surface of plastic dishes coated with cellular microexudate from CE or ME cells with 0.1% crystalline trypsin for 10 min at 37°C, followed by several washes with PBS to elute any residual active enzyme (22), abolished the ability of microexudates to enhance the growth of low-density homotypic cell populations.

To determine whether the enhanced growth of low-density cell populations seeded onto microexudate was due in part to the release of growth-stimulating macromolecules from the microexudate into the culture medium, in a situation analogous to conventional "conditioned" medium, plastic dishes coated with microexudates were incubated with culture medium without cells for 5 days at 37°C. The medium harvested from these dishes was then used to treat the surface of new untreated plastic dishes. Cells were then seeded at low densities (1×10^4 cells/dish) onto the surface of these dishes and cultured for 6 days in the medium harvested as described above. Comparison of the growth of CE cells in these dishes with similar low-density CE cell populations seeded onto untreated plastic dishes failed to reveal any significant differences in the number of cells after 6 days. These results suggest that the enhanced cell growth at low densities induced by cellular microexudates

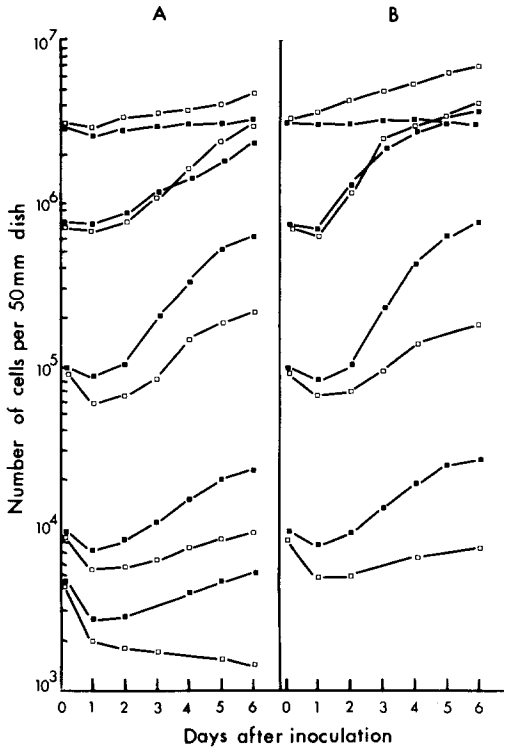

FIGURE 2 Growth of chick embryo (A) and mouse embryo cells (B) seeded at different densities onto normal plastic dishes (□——□) or onto similar dishes coated with microexudates from exponentially growing homotypic cells (density: 8×10^4 cells/cm²) (■——■). Each point represents a mean value derived from cell counts on four separate 50-mm dishes.

requires direct contact interaction between the inoculated cells and the microexudate.

Specificity of Growth Enhancement Induced by Cellular Microexudates

In addition to the enhanced growth of low-density CE and ME cell populations produced by seeding them onto homotypic microexudates, similar growth enhancement was obtained when low-density ME cell populations were inoculated onto substrates coated with intraspecific or interspecific heterotypic cellular microexudates (Fig. 3).

Enhancement of Cell Growth at Low Densities by Cellular Microexudates: Influence of Microexudate Donor Cell Density

The enhanced growth of low-density cell populations seeded onto homotypic cellular microexudates reported in previous sections was achieved with microexudates deposited by actively growing subconfluent or near-confluent cell cultures. In contrast, microexudates derived from cells of the same type in confluent stationary phase cultures did not enhance the growth of low-density cell populations (Table III). As shown in Table III, low-density ME or CE cell populations inoculated onto homotypic microexudates from subconfluent cells in their exponential growth phase ($8–9 \times 10^4$ cells/cm²) showed significantly greater growth than low-density cell populations on microexudates from dense confluent cultures of phase cells or seeded onto untreated normal culture dishes.

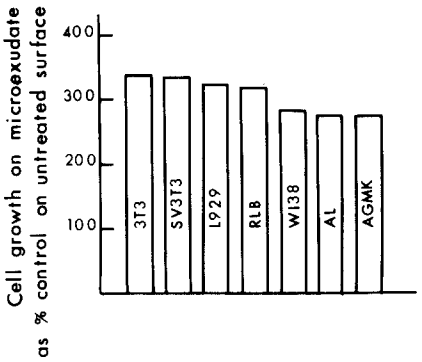

FIGURE 3 The growth of mouse embryo cell populations seeded at low densities (1×10^4 cells/dish) on microexudates from exponentially growing heterotypic cells. Cell growth on microexudate-coated substrates is expressed as a percentage of the growth of control cell cultures seeded at identical densities on to untreated plastic dishes. The microexudate donor cell populations were used at the following densities (number of cells/cm² $\times 10^4$): 3T3 5.2; SV3T3, 15.8; L, 39.0; RLB, 4.9; W138, 15.3; AL 10.5; and AGMK, 4.7.

TABLE III

*The Effect of Microexudates from Logarithmic Phase Cell Cultures and Dense Stationary Phase Cultures on the Proliferation of Homotypic Cells Seeded at Low Density**

Cell type	Density of microexudate donor cells‡	Cell number 6 days after inoculation	Ratio of number of cells at 6 days to number of cells in original inoculum
	cells/cm² × 10⁴	*cells/dish*	
ME	Untreated control§	9.02×10^3	0.90
ME	8.1	3.41×10^4	3.41
ME	40.3	7.14×10^3	0.71
CE	Untreated control§	1.42×10^4	1.42
CE	8.6	3.37×10^4	3.37
CE	37.4	0.60×10^3	0.60

* The results represent mean values derived from four separate experiments (1×10^4 cells/50-mm dish).
‡ Mean values derived from cell counts on 10 replicate 50-mm Petri dish cultures in each experiment.
§ Control cultures were seeded in untreated 50-mm plastic Petri dishes.

Although the results in Table III demonstrate that microexudates from confluent stationary phase cultures did not enhance cell growth, data obtained in the following experiments suggest that such microexudates can actually inhibit growth of homotypic cells. This growth-inhibitory effect was detected in experiments in which ME and CE cells were seeded onto microexudates at initial densities higher than those used in previous experiments. These higher seeding densities were sufficient under normal culture conditions in untreated plastic Petri dishes to permit rapid cell growth and the formation of confluent monolayers 3–4 days after inoculation. Measurement of the proliferation of cells seeded at such densities on microexudate-coated substrates revealed that microexudates from dense stationary phase homotypic cell cultures produced marked growth inhibition (Table IV). In contrast, microexudates deposited by actively growing logarithmic phase cells of the same type had no inhibitory effect on the growth of the inoculated cell population, which grew rapidly to achieve a saturation density similar to that in

control cell populations seeded in untreated plastic Petri dishes (Table IV).

The results in Table IV indicate that the growth inhibitory effect of microexudates from dense stationary phase cultures is not confined merely to very low-density cell populations, which would display slow growth rates even under normal culture conditions, since a similar inhibitory effect is exerted on cells seeded at densities that would normally be sufficient to allow rapid growth.

Further observations on the proliferation of a range of human, mouse, monkey, and rat diploid cell strains seeded onto homotypic cell microexudates revealed that microexudates from stationary phase cultures were uniformly inhibitory to the growth of the inoculated cells, while microexudates from logarithmic phase cultures had no significant effect on cell proliferation (Table V).

These results indicate that inhibition of the growth of diploid cells by microexudates from stationary phase cultures may be a relatively wide-spread phenomenon.

Detailed information of the inhibitory effect of cellular microexudates from stationary phase cultures on the growth of heterotypic cells, together with data on the effect of microexudates from normal cells on the proliferation of tumor cells, will be presented in a subsequent paper.

TABLE IV

*The Effect of Microexudates from Logarithmic Phase Cell Cultures and Dense Stationary Phase Cultures on the Proliferation of Homotypic Cells Seeded at Medium Densities**

Cell type	Density of microexudate donor cells‡	Cell number 6 days after inoculation	Ratio of number of cells at 6 days to number of cells in original inoculum
	cells/cm × *10⁴*	*cells/dish*	
ME	Untreated control§	2.52×10^6	5.04
ME	8.4	2.64×10^6	5.28
ME	39.7	5.63×10^5	1.12
CE	Untreated control§	2.90×10^6	5.80
CE	8.6	2.72×10^6	5.44
CE	38.2	5.76×10^5	1.15

* The results represent mean values derived from four separate experiments (5×10^5 cells/50-mm dish).
‡ Mean values are derived from cell counts on 10 replicate 50-mm Petri dish cultures in each experiment.
§ Control cultures were seeded in untreated 50-mm plastic Petri dishes.

TABLE V

*The Effect of Homotypic Cell Microexudates from Logarithmic Phase and Dense Stationary Phase Cultures on the Proliferation of Mouse (3T3), Human (AL; W138), Rat (RLB), and Monkey (AGMK) Diploid Cell Strains**‡

Cell type	Density of microexudate donor cells‡	Cell number 6 days after inoculation§	Ratio of number of cells at 6 days to number of cells in original inoculum
	cells/cm² × *10⁴*	*cells/dish*	
3T3	Untreated control‖	1.71×10^6	5.72
	4.9	1.90×10^6	6.33
	10.1	3.42×10^5	1.14
AL	Untreated control‖	4.07×10^6	4.52
	10.2	3.93×10^6	4.37
	19.3	8.64×10^5	0.96
W138	Untreated control‖	4.35×10^6	5.80
	15.8	4.19×10^6	5.59
	21.2	8.03×10^5	1.07
RLB	Untreated control‖	1.58×10^6	3.95
	4.9	1.39×10^6	3.48
	8.2	4.08×10^5	1.02
AGMK	Untreated control‖	1.55×10^6	3.88
	4.5	1.73×10^6	4.33
	8.2	5.16×10^5	1.29

* The results represent mean values derived from three separate experiments.
‡ Cells were seeded at the following densities (number of cells/50-mm dish): 3T3, 3×10^5; AL, 9×10^5; W138, 7.5×10^5; RLB, 4×10^5; and AGMK, 4×10^5.
§ Mean values were derived from cell counts on 10 replicate 50 mm-Petri dish cultures in each experiment.
‖ Control cultures were seeded in untreated 50-mm plastic Petri dishes.

Enhancement of Cell Growth at Low Densities by Cellular Microexudates: Comparison with the Effect of Conditioned Medium or Feeder Cells

Numerous previous studies have shown that the growth of low-density cell populations can be enhanced by cultivation in "conditioned medium" (CM) obtained from dense-growing cell cultures of

the same type (21, 30) or by plating onto "feeder" cell layers of the same (10, 28, 33) or a different cell type (36, 41). It was considered pertinent therefore to compare the growth enhancement of low-density CE or ME cell populations produced by homotypic microexudates with that produced by CM or homotypic feeder cell layers.

The results shown in Fig. 4 indicate that both CM and X-irradiated feeder cell layers induce significantly greater growth enhancement of low-density cell populations than homotypic microexudates.

DISCUSSION

The present experiments have demonstrated that mammalian cells cultured on solid substrates in vitro deposit microexudates of cellular macromolecules that remain attached to the substratum when the cells are detached. The results described here for radioactively labeled cells indicate that the type of cellular macromolecules deposited on the substratum varies significantly between different cell types and between cells of the same type at different population densities. These findings complement previous observations that have shown that the rate of synthesis and the amount of microexudate deposited by different cell types vary significantly (25). The present results confirm and extend the observation of Yaoi and Kanaseki (45) that cell growth at low densities can be enhanced by inoculation onto substrates coated with macromolecular material deposited by actively growing cells of the same type. The work described here has shown that similar growth enhancement can also be induced by microexudates from heterotypic cells. Enhanced growth of low-density cell populations induced by cellular microexudates is not as efficient, however, as that produced by cultivation of similar small numbers of cells in conditioned medium or in contact with feeder cell layers.

The physiological state of the cells used to provide microexudate has been found to influence the growth behavior of cells inoculated onto substrates coated with microexudates. Microexudates deposited by subconfluent cell cultures in their exponential growth phase are required for optimum enhancement of the growth of low-density cell populations, since microexudates deposited by confluent cells of the same type in the stationary growth phase exert an inhibitory effect on cell growth, irrespective of the density of the cell population inoculated onto the microexudate. The present findings on differences in the effect of microexudates from confluent and subconfluent donor cells on cell growth have certain similarities with the observations of Bolund et al. (3), who found that the cytochemical changes in the nuclei

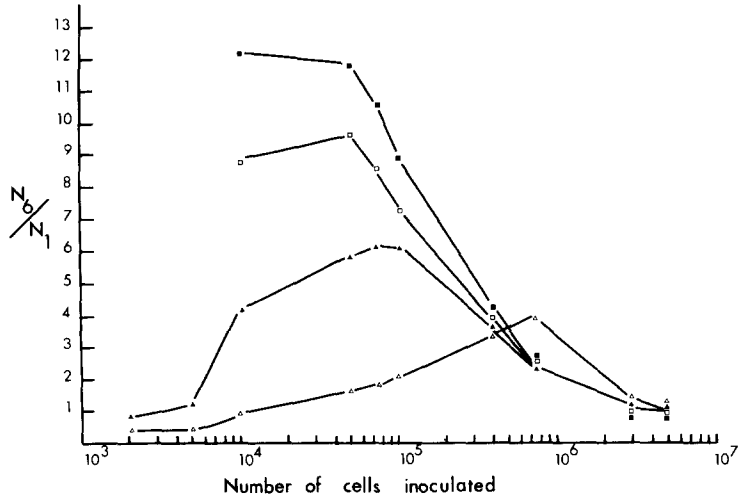

FIGURE 4 Growth of chick embryo cells seeded at different densities on dishes coated with microexudate from exponentially growing homotypic cells (density 8×10^4 cells/cm^2) (▲——▲), or onto X-irradiated chick embryo feeder cell layers (□——□), or cultivated in conditioned medium (■——■). Control cell cultures (Δ——Δ) were seeded on untreated plastic dishes. Cell growth is expressed as a ratio of the number of cells present 6 days after inoculation (N_6) to the number of cells in the original inoculum (N_1). Each point represents a mean value derived from cell counts on three separate dishes.

of hen erythrocytes accompanying "activation" of nucleic acid synthesis could be rapidly induced by plating cells onto glass substrates coated with microexudates from exponentially growing HeLa cells or mouse A9 fibroblasts, but that at high cell densities these nuclear changes did not occur. A further similarity between the present study and that of Bolund and colleagues is that the growth-enhancing effects of the microexudates could be abolished by brief trypsinization.

It is of interest to note, however, that optimum enhancement of cell growth at low densities was produced by microexudates deposited by subconfluent donor cultures in which 30–40% of the substratum was free of attached cells. This finding suggests that the deposition of microexudate on the substratum is not confined solely to the area immediately beneath attached cells, and that this material is able to spread over the substratum in the intercellular areas. Direct support for the presence of microexudates in the intercellular areas of subconfluent cell cultures has been provided by ellipsometry (26, 32) and immunofluorescence (3). The ability of cellular macromolecules with growth-promoting properties to spread laterally in the plane of the surface from attached cells has also been proposed, from observations on the social behavior of cells in the vicinity of wounds inflicted in confluent monolayers (35), and from studies on the growth-enhancing effects of feeder cell layers on low-density cell populations separated from the feeder cells by increasing distances (30, 45).

The mechanism by which microexudates derived from exponentially growing cell cultures enhance cell growth at low densities remains to be established. The use of EDTA or trypsin, or both, to detach cells from substrata results in the removal or peripheral components from cells (6–8, 17, 20, 22, 38) that are resynthesized over a period of 1–8 h (2, 13, 15, 17, 19, 25, 26). The removal of these peripheral components creates abnormal permeability fluxes across the plasma membrane, and cells remain "leaky" until the surface components are resynthesized. In addition, the increased permeability of the cells reduces their proliferative capacity, and this process is exacerbated in low-density cell populations seeded in relatively large volumes of culture medium (11). A possible interpretation of the enhanced growth of low-density cell populations when seeded on microexudates is that the microexudates limit the permeability deficit of the seeded cells, enabling them to equilibrate more rapidly with their culture environment and start dividing more quickly than similar cells on untreated substrates.

Since the growth rate of low-density cell populations seeded onto microexudates is also affected by whether the microexudate donor cells were growing or were in stationary phase, it seems likely that, in addition to their possible effects in limiting the abnormal permeability of newly seeded cells, microexudates also contain components that can influence cell growth per se. In this respect, it is pertinent to note that previous studies on the interaction between intact cells have shown that diploid cells in the stationary phase can inhibit or greatly retard the growth of other diploid cells seeded onto them, though replicating cells of the same type do not affect the growth of the seeded cell population (10).

The possible similarities between the growth-inhibitory properties of microexudates and those of intact cells suggest that these properties in intact cells may be mediated in part by specific functional determinants in the cell periphery, which are also present in microexudates of cell surface macromolecules. Little information is available, however, on the structural components in the cell periphery that may be involved in those aspects of cell growth regulation that appear to be mediated by cell-to-cell contact phenomena. Data presented here have shown that the gross macromolecular composition of microexudates differs significantly between cells in sparse actively replicating cultures and those in confluent stationary cultures. A more detailed characterization of the chemical composition of cellular microexudates with different cell growth inhibitory properties might therefore offer an opportunity for identifying cell surface determinants that may regulate cell growth.

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