

## An Inhibitor of Animal Cell Growth Increases Cell-to-Cell Adhesion

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**ABSTRACT** The interactions of both normal and transformed cells with their environment is mediated to a large extent by the cell surface. Succinylated concanavalin A (succinyl-Con A) is a nontoxic and nonagglutinating derivative of the jack-bean lectin concanavalin A. Succinyl-Con A, presumably through an interaction with the cell surface, reversibly inhibits the growth of normal cells and restores a normal growth phenotype to transformed cells. Whereas at high cell densities migration was inhibited, it turned out that at low cell densities where cells are not in contact with each other, cell movement was not affected by succinyl-Con A. Together with some additional observations, this suggests that this lectin derivative increases cell-to-cell adhesion in culture and thereby may influence cell migration. An increase in cell-to-cell adhesion by this lectin derivative may not be brought about simply by physically linking cells together. It occurs after a lag time, possibly by inducing surface changes. The relationship between cell adhesion in culture, cell movement, and cell growth is discussed.

Succinylated concanavalin A (succinyl-Con A) is a divalent, nonagglutinating and nontoxic derivative of the tetravalent jack-bean lectin concanavalin A (Con A) (19). Succinyl-Con A reversibly inhibits the growth of 3T3 cells and restores a normal growth phenotype to SV40 transformed 3T3 cells (i.e., inhibition of anchorage independent growth and, on a solid substratum, induction of a quiescent population of cells accumulated in the G<sub>1</sub> phase of the cell cycle) (25, 26). Succinyl-Con A-induced growth inhibition involves binding to carbohydrate moieties and is independent of serum factors and substratum availability (5). An important aspect of succinyl-Con A-induced growth inhibition is that for any given concentration of succinyl-Con A the growth of a population of cells ceases at a specific density, independent of the initial, plating density. That is, populations of cells plated at different initial densities but treated with the same concentration of succinyl-Con A will become quiescent at the same final density. These results suggest that a minimum cell number and, therefore, some form of cell-cell interaction are necessary for succinyl-Con A-induced growth inhibition.

In sparse culture, cells treated with succinyl-Con A appear morphologically identical to control cells. At quiescence, however, in contrast to control cells that form a confluent monolayer, cells treated with succinyl-Con A become quiescent at subconfluent levels and collect in small patches, leaving large

areas of the substratum devoid of cells (26). These observations suggested that succinyl-Con A might, in some way, be altering cell migration and/or cell-cell adhesion. Due to our interest in succinyl-Con A as a reversible inhibitor of cell growth, we chose to analyze the effects of succinyl-Con A on cell migration and cell-cell adhesion in conditions that would most closely approximate the conditions previously used in our growth studies.

### MATERIALS AND METHODS

Succinyl-Con A was prepared as described previously (5). Mouse embryo 3T3 and SV40 3T3 cells were grown in Falcon tissue culture dishes (Falcon Labware, Oxnard, Calif.) in Dulbecco's modified Eagle's (DME) medium supplemented with calf serum (Gibco Laboratories, Grand Island Biological Co., Grand Island, N. Y.).

### *Migration in Dense Culture*

For migration assays according to Burk, 3T3 cells were grown to monolayers in 35-mm dishes (7, 8). SV40 3T3 cell monolayers were obtained by seeding  $2 \times 10^6$  cells into 35-mm dishes containing 2 ml of DME plus 10% calf serum. After 1 d the medium was changed to DME plus 2% calf serum. 2 d later the monolayers were wounded with a razor blade and the medium was changed to DME plus 2% calf serum with or without succinyl-Con A; 24 h later the cells were fixed with ethanol and stained with Giemsa's. The cells that had migrated into the cell-free space were counted. All samples were determined at least in duplicate.

## Time-lapse Cinemicroscopy in Sparse Culture

Time-lapse cinemicroscopy was done using a Wild M 40 inverted microscope to which a Paillard Bolex H camera was connected. A Paillard-Wild MBF-B Variotimer was connected to the camera and exposures were made at 3-min intervals on Kodak Plus-X film. For monitoring the cell migration rate, a Bolex 521 projector was used and the positions of cell nuclei were marked every 50 frames. The average migration rate was calculated from about 30 individual cells. Tissue culture was done as described for the Burk assay.

## Measurement of Migration from Tracks through Gold Particles

The gold particle assay was used as described by Albrecht-Buehler (2). The glass cover slips used for the assay were first coated with collagen. Then these glass cover slips in 15-mm Limbro-Multiwells were covered with colloidal gold, and  $0.75-1.0 \times 10^3$  3T3 or SV40 3T3 cells were seeded on top of the gold. Cells were incubated in 0.5 ml of medium for 24 h as described for the Burk assay.

## RESULTS

The effect of succinyl-Con A on the ability of cells to migrate in dense culture was assayed using a known procedure described by Burk (7, 8). At various times after creating a sharp boundary in a confluent monolayer with a razor blade the cultures are fixed and stained, and migration is scored as the number of nuclei that have crossed the boundary. Succinyl-Con A inhibits the migration in dense cultures of 3T3 and SV40 3T3 cells in a dose-dependent manner. Inhibition of migration increases as the concentration of succinyl-Con A increases (Fig. 1).

Succinyl-Con A-induced inhibition of migration probably involves binding to carbohydrate moieties because this effect can be prevented by and reversed by the addition of 10 mM  $\alpha$ -methyl-D-mannopyranoside, a Con A hapten sugar (data not shown). Also, succinyl-BSA, containing almost twice as many succinyl groups per molecule, has no inhibitory effect on cell migration, demonstrating that inhibition of migration as tested in this assay is not simply due to the negative charges of the succinate groups (data not shown).

Serum has been shown to contain factors that are stimulatory as well as necessary for cell migration in general (23). However, increasing the concentration of serum in the medium to as much as 25% does not reduce the extent of succinyl-Con A-induced inhibition of migration in this assay using dense cultures of 3T3 cells (Fig. 2). Because the extent to which cell

migration is inhibited is independent of serum concentration, the inhibitory effect is not likely to be due to binding and/or inactivation of migration factors in the medium. Unlike 3T3 cells, SV40 3T3 cells more readily respond to increased serum concentrations by an increase in growth rate. The migration assay in dense culture for SV40 3T3 cells has therefore been carried out at low serum concentrations only, to avoid extensive stimulation of cell growth in the monolayer.

Inhibition of cell migration by succinyl-Con A in dense culture as measured by the Burk assay could be due to either an inhibition of the motility of single cells by succinyl-Con A and/or a succinyl-Con A-induced increase in cell-cell adhesion. To distinguish between these two possibilities, the migration rates of single cells in sparse culture were determined by two different methods.

Table I shows the average migration rates of 3T3 and SV40 3T3 cells measured using time-lapse cinemicroscopy. Both cell types migrate at essentially the same rate, and the rate of migration is not changed by the addition of succinyl-Con A to the medium. It is important to note that the cells were followed only as long as they had no contact with neighboring cells. Daughter cells that had gone through mitosis and cells that had collided with one another were often sticking together. The migration of such cells was impaired, and they were, therefore, no longer monitored.

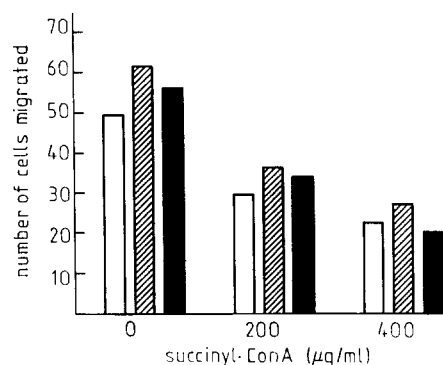


FIGURE 2 Effect of serum on succinyl-Con A induced inhibition of migration of 3T3 cells in dense culture. Migration in dense culture was assayed in Fig. 1. (▨) 5% serum; (□) 10% serum; (■) 25% serum.

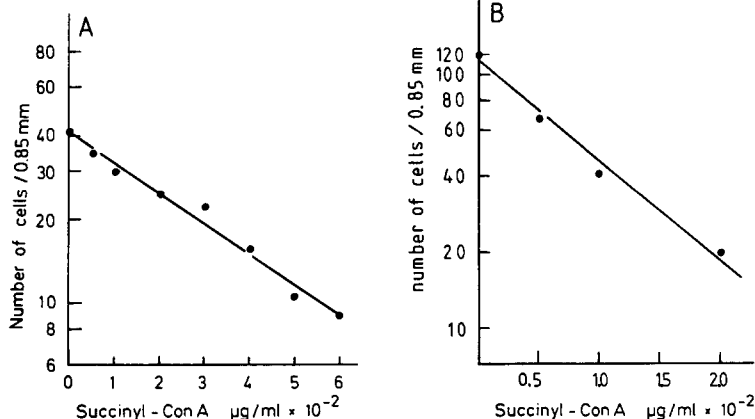


FIGURE 1 Effect of succinyl-Con A on cell migration determined by the Burk assay. (A) Number of migrating 3T3 cells. (B) Number of migrating SV40 3T3 cells.

TABLE I

Migration Rates of 3T3 and SV40 3T3 Cells in Sparse Culture Determined with Time-lapse Cinemicroscopy

Succinyl-Con A	3T3 Cells	SV40 3T3 Cells
$\mu\text{g/ml}$	$\mu\text{m/h} \pm \text{SD}$	$\mu\text{m/h} \pm \text{SD}$
0	$13.1 \pm 4.7$	$13.6 \pm 0.6$
200	—	$11.0 \pm 6.3$
500	$11.1 \pm 6.5$	—

TABLE II

Migration Rates of 3T3 and SV40 3T3 Cells in Sparse Culture Determined by Following Phagokinetic Tracks

Succinyl-Con A	3T3 Cells	SV40 3T3 Cells
$\mu\text{g/ml}$	$\mu\text{m/h} \pm \text{SD}$	$\mu\text{m/h} \pm \text{SD}$
0	$11.9 \pm 3.4$	$3.7 \pm 1.4$
50	$9.5 \pm 2.6$	$3.5 \pm 1.4$
100	$10.5 \pm 2.6$	$3.2 \pm 1.2$
200	$9.9 \pm 3.4$	$3.3 \pm 1.0$
400	$11.8 \pm 4.3$	$2.9 \pm 0.9$
1000	$8.7 \pm 3.6$	$2.8 \pm 0.7$

In another assay, the migration rate was measured by determining the length of phagokinetic tracks left behind by cells migrating on a surface coated with gold particles (2). Table II shows the average migration rates measured over a 24-h period. No significant decrease in the migration rates of either 3T3 or SV40 3T3 cells was observed after succinyl-Con A treatment. The rates of migration of 3T3 cells were found to be comparable to those observed with time-lapse cinemicroscopy. However, SV40 3T3 cells migrate at a considerably slower rate on a surface covered with gold particles compared with their migration rate on tissue culture plastic. In the conditions necessary for the phagokinetic track assay, our SV40 3T3 cells are not as well spread in comparison to cells on untreated glass or plastic surfaces, suggesting that cell-substratum adhesion was reduced (15). Ali and Hynes have demonstrated that increasing the cell-substratum adhesion by the addition of fibronectin increases cell migration (4). Thus, the decreased rate of migration of untreated SV40 3T3 cells in the gold particle assay, compared to the rate determined by time-lapse cinemicroscopy, is most likely due to decreased cell-substratum adhesion in those assay conditions. More significant, however, is the finding that even very high doses of succinyl-Con A have no inhibitory effect on the migration rates of single cells (Tables I and II), demonstrating that succinyl-Con A does not decrease cell-substratum adhesion as measured by these assay systems.

## DISCUSSION

In the past few years much attention has been focused on the migratory behavior of tumor cells in culture because their malignancy is in part due to their invasiveness into neighboring tissues (1, 6, 11, 14, 30, 37, 41). The cytoskeleton, as well as the cell surface, is thought to influence cell migration (4, 32, 33, 42, 47, 48).

The data presented here lead to the suggestion that succinyl-Con A inhibits migration in dense culture by increasing the lateral adhesion between both 3T3 and SV40 3T3 cells. The parameters that determine the adhesiveness of one cell to another in culture are not well understood, and the information available to date suggests that cell recognition and adhesion is a multistep process (9, 16, 28). Furthermore, most adhesion

assays measure cell adhesion between cells in suspension or between suspended cells and cell monolayers. We felt therefore that adhesion assays where at least one of the cell partners is free and not attached to a substratum may not reflect the actual situation in a tissue culture dish or eventually in the tissue. For this reason, as well as the fact that succinyl-Con A does not promote aggregation in suspension of the cell lines used in this study, we studied cell behavior *in situ*.

Because dense cultures might be expected to have a more elaborate extracellular matrix than sparse cultures, it is possible that succinyl-Con A decreases cell motility in dense culture by binding cells to the extracellular matrix. We believe that this is not occurring. Besides the reduced movement in dense cultures, three additional morphological observations support the notion that succinyl-Con A promotes increased cell adhesion between neighboring cells in culture: (a) Within 1 d after adding succinyl-Con A to sparse cells in culture they form clustered mini-monolayers (26). Few single cells are observed. Most cells stick tightly to their neighbors. (b) Time-lapse cinemicroscopy clearly shows that pairs of cells in contact with one another, both as a result of collision and as a consequence of mitosis even in sparse culture, are highly adhesive in the presence of succinyl-Con A and can no longer pull apart from each other. (c) In studies using scanning electron microscopy, we have observed a dramatic increase in the number of adhesive bridges (microvilli-like) connecting the surface of neighboring cells after succinyl-Con A treatment (Fig. 3).

The mechanism by which succinyl-Con A increases lateral adhesion between cells is unknown. Succinyl-Con A is a poor agglutinin when tested on cells in suspension, but it cannot be ruled out that it may act as a crosslinker of flattened cells on a solid substratum. Alternatively, succinyl-Con A may induce a change in the plasma membrane, leading to an increase in cell-cell adhesion. The observation that cells that were blocked from migrating out of a wounded monolayer by the addition of succinyl-Con A do not readily reinitiate migration after withdrawing the lectin seems to favor the latter hypothesis. The same lag was observed in time-lapse movies. Furthermore, the changes observed in scanning electron microscopy studies could not be seen during the first 3 h after lectin addition.

Low concentrations of humoral factors (for example, hormones and serum growth factors) and/or an inadequate substratum availability will limit the growth of nontransformed cells (15, 17, 18, 21, 24, 29, 35, 39). However, in conditions of excess humoral factors and an appropriate substratum, the growth of nontransformed cells is limited by the density of the population (12, 13, 34, 38, 40). Thus, the regulation of proliferation of animal cells involves an interplay between positive growth effectors such as humoral factors and a suitable substratum and negative growth effectors such as high cell density and possibly cellular growth inhibitory factors.

The mechanism of density-dependent inhibition of growth is unknown. It has been proposed that this phenomenon could simply be related to a decrease in the accessibility to cells of growth-promoting factors in the medium, but recent experiments suggest that other mechanisms may be involved (36, 43).

Another theory is that specific cell-cell contacts formed between surface components of neighboring cells may be responsible for density-dependent growth inhibition (22, 34, 40, 44, 45). Two different studies have shown that simply increasing cell adhesion is not sufficient to induce growth inhibition, suggesting that, if cell-cell interactions are functionally involved in growth regulation, these interactions are probably

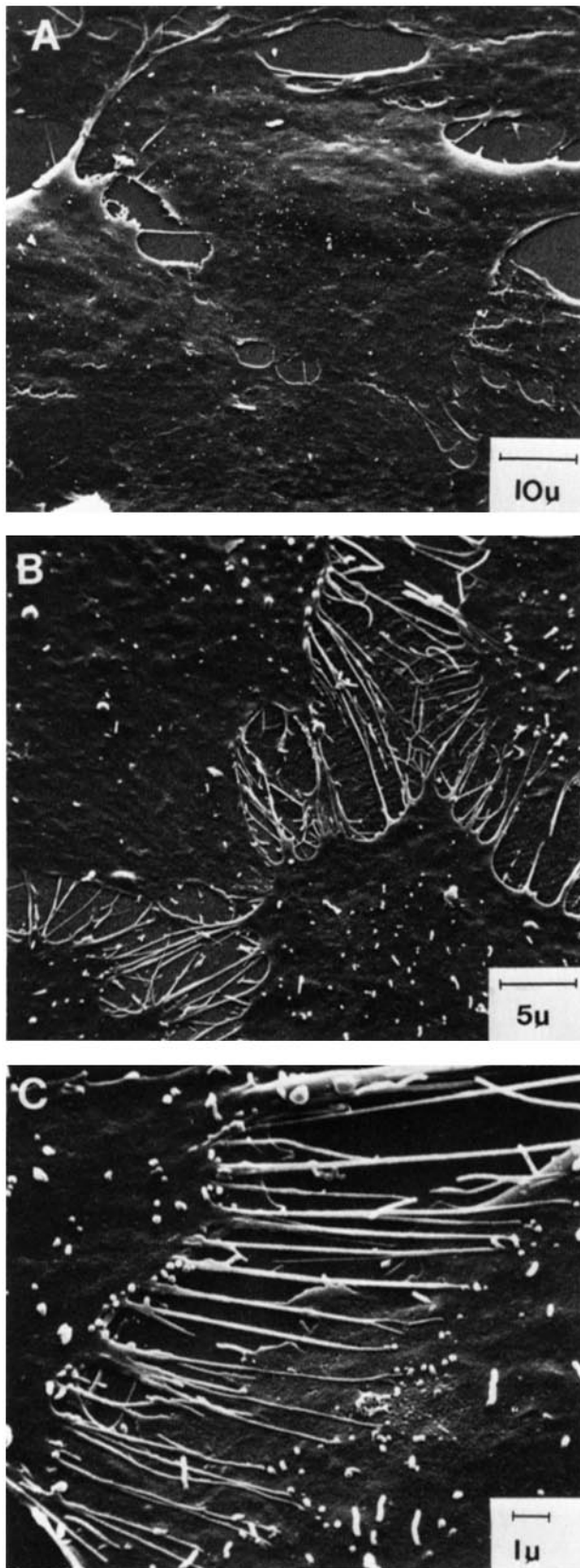


FIGURE 3 Scanning electron micrographs of 3T3 cells in the presence and absence of succinyl-Con A. A—arrested control cultures  $\times 1200$ ; B—cultures arrested with  $500 \mu\text{g/ml}$  succinyl-Con A  $\times 2400$ ; C—cell junction in a culture arrested with  $500 \mu\text{g/ml}$  succinyl-Con A  $\times 6000$ .

mediated by a specific class of cell surface components. (a) Fibronectin, which restores a normal morphology to transformed cells, increases both cell-cell adhesion and cell-substratum adhesion but has no growth inhibitory properties (3, 10, 47, 48). (b) An antiserum prepared against surface membranes of transformed BHK21 cells resulted in an increase in cell-cell adhesion and a decrease in cell-substratum adhesion (46). The growth rate of cell cultures in the presence of this antiserum was not reduced and the cells grew to the same high densities as untreated cultures. In other studies, Hakomori has reported that monovalent antibodies to either ganglioside  $\text{GM}_1$  or ganglioside  $\text{GM}_3$  inhibit the transformed phenotype but do not inhibit the growth of cells on a solid substratum, whereas divalent (i.e., succinyl) Con A or monovalent antibodies to globoside inhibit growth but do not inhibit the transformed phenotype (20).

The hypothesis that specific cell-cell contacts are functionally involved in density-dependent inhibition of growth has gained support recently from two different studies. (a) Plasma membrane-enriched fractions of cultured cells can induce growth inhibition in subconfluent populations of cells capable of responding to density-dependent growth inhibition (22, 44, 45). Growth inhibition by cell surface membranes is reversible and occurs in the presence of excess serum factors. (b) Experiments studying the effects of microtubule-dissociating agents on cell growth indicate that "there is a distinct state of contact inhibition of cell growth that is different from simple nutrient depletion" (13).

Transformed cells are unresponsive to growth regulation by nutrient depletion, substratum availability, and/or population density. Transformed cells treated with succinyl-Con A have regained anchorage-dependent growth and respond to growth regulation by humoral factors (27). These data imply a contact-dependent phenomenon, which is induced by succinyl-Con A and is not directly related to humoral factors, that can restore normal growth to transformed cells.

The effects of increasing concentrations of succinyl-Con A on growth inhibition are similar to the effects on cell migration; there is a linear relationship between the log of percent inhibition and increasing succinyl-Con A concentrations (compare Fig. 1A to Fig. 1A in reference 26, and Fig. 1B to Fig. 1 in reference 5). Quantitatively, a given concentration of succinyl-Con A is approximately twice as effective at inhibiting cell migration as it is at inhibiting cell growth (for example, the concentration of succinyl-Con A necessary to reduce the final density of a cell culture by 25% will inhibit migration by 50%). Although there is strong circumstantial evidence suggesting a correlation between succinyl-Con A-induced growth inhibition and increased lateral cell-cell adhesion, a functional link between these two phenomena remains to be established. Succinyl-Con A promises to be an effective reagent for identifying cell surface macromolecules functionally involved in growth regulation, and for investigating the possible role of specific cell-cell contacts in the mechanism of density-dependent growth inhibition.

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