Density-Dependent Growth Inhibiting Interactions between 3T3 and SV40-3T3 Cells in Mixed Cultures

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3T3 cells are shown to reduce SV40-3T3 cell population growth in a density-dependent manner in co-cultures of 3T3 and SV40-3T3 cells. The development of this inhibitory activity roughly parallels the development of density-dependent inhibition of growth in homogeneous 3T3 control cultures. The extent of reduction of SV40-3T3 growth can be manipulated by pretreatment of 3T3 cells with a high serum concentration. SV40-3T3 growth rates are reduced by factors between 10 and 20 under optimum inhibitory conditions as compared to SV40-3T3 growth in control cultures.

Introduction

Regarding the mechanisms which lead to density dependent inhibition of growth [*e. g.* 2–4, 6, 12, 13, 15, 19] in normal cell populations and the disturbance of these mechanisms by tumorigenic transformation [*e. g.* 17, 18] it is an important question to what extent the growth of transformed cells can be influenced by normal cells. Several reports have appeared in the literature describing the establishment of growth inhibiting interactions between normal and transformed cells in mixed cultures under conditions where the normal cells reached their saturation density [1, 5, 8, 10, 11, 14]. The extent of such inhibiting interactions depends on both, cell types and transforming agents used in such experiments [1, 5, 7, 8].

We were interested in the question of how such inhibitory interactions betwen normal and transformed cells depend on the cell number ratio of the two kinds of cells and on conditioning the cells in various ways before seeding the transformed cells onto the normal ones. In earlier work [20] it was shown that pretreatment of 3T3 mouse fibroblasts with 10% serum made the cells more sensitive or active towards density-dependent inhibition of growth at 2.5% serum. In the same work, density-dependent death of 3T3 cells could be shown to occur after serum reductions at cell densities higher than the saturation density at the reduced serum concentration. In the present work, we investigated how such treatments of 3T3 cells influence the interaction of 3T3 and SV40-3T3 cells in mixed culture.

In addition, we report on the lack of effects on this interaction of treatments of SV40-3T3 cells with various trypsin concentrations in the presence and absence of divalent cations.

Materials and Methods

Cell strains

Swiss 3T3 cells [16] and their SV40-virus transformed counterparts (line 101) are from stocks originally provided by Dr. M. M. Burger, Basel. Stem cultures of both cell strains were grown in Greiner plastic Petri dishes in antibiotica-free medium (Dulbecco's modification of Eagle's minimal essential medium (DMEM), Flow Laboratories) at two different concentrations (2.5%, 10%) of new-born calf serum from Gibco Bio-Cult Ltd. In all stem cultures the medium was changed every two days. 3T3 cells were passaged at sub-confluency about every four days. SV40-3T3 cells were passaged depending on the serum concentration once or twice a week. All cultures were kept in incubators at 37 °C in a humidified atmosphere of 10% CO_2 in air.

Cell counting

Cell numbers per plate were determined by counting the trypsinized cells in suspensions of appropriate dilution with an automatic cell counter (Coulter, system ZF). Special care was taken to ensure equal and uniform cell density at the start of an experiment in all culture dishes belonging to one growth curve. Therefore, cells were seeded from stirred suspensions by means of a hand-driven gently-working dispensor. Directly after seeding the plates were placed as horizontally as possible in the

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incubator. With these precautions the counts in triplicate seedings differed by less than 5% from each other at the time of seeding and by less than 10% throughout the whole experiment. Purified trypsin from bovine pancreas (Boehringer) has been used for detachment of cells from the plates. For seedings the trypsin concentration was 1 or 2 mg per 100 ml of Earle's salt solution buffered by 10 mM Tris (hydroxymethyl) aminomethane (TRIS) at pH 7.4. For cell countings the cells were detached by means of 6 mg trypsin per 100 ml.

Results

Dependence in mixed populations of SV40-3T3 cell multiplication on 3T3 cell density and pretreatment of 3T3 cells by high serum concentration

In the following experiments we have tried to find out whether interactions exist between 3T3 and SV40-3T3 cells in mixed cultures and how such interactions depend on the state of the 3T3-cell population. At first, we want to describe these different 3T3-cell population states. In Fig. 1, the population development of 3T3 cells has been recorded at two different serum concentrations: 2.5% (lower solid line) and 10% (upper solid line). The dashed lines indicate population development after sudden serum reductions from 10% to 2.5% at various cell densities. In order to ensure constant environmental parameters, the medium was renewed daily in all cultures throughout the experiment. The saturation densities at the two serum concentrations differ by a factor of about 2.2, and they are approached by very different kinetics of growth retardation. This is borne out clearly, if the log growth rate $R = d\ln N/dt = \ln 2/T_d$ is plotted against time or cell density, (T_d: density dependent population doubling time; N: cell density).

For Fig. 2 T_d has been taken from the slopes of the intercepts between measured points in Fig. 1 under the approximation of constant logarithmic growth between two consecutive data points. Fig. 3 shows $1/T_d$ *versus* normalized cell density N/N_m, where N_m is the maximum density reached at a fixed serum concentration. It can be seen from these dia-

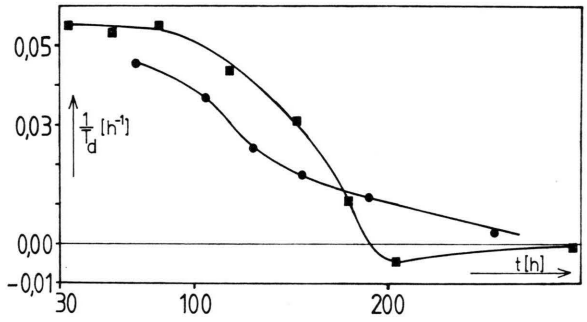

Fig. 2. 3T3 cell population growth rate in dependence on time at 2.5% (circles) and 10% (squares) serum. T_d is the population doubling time. Data are taken from Fig. 1 as described in the text.

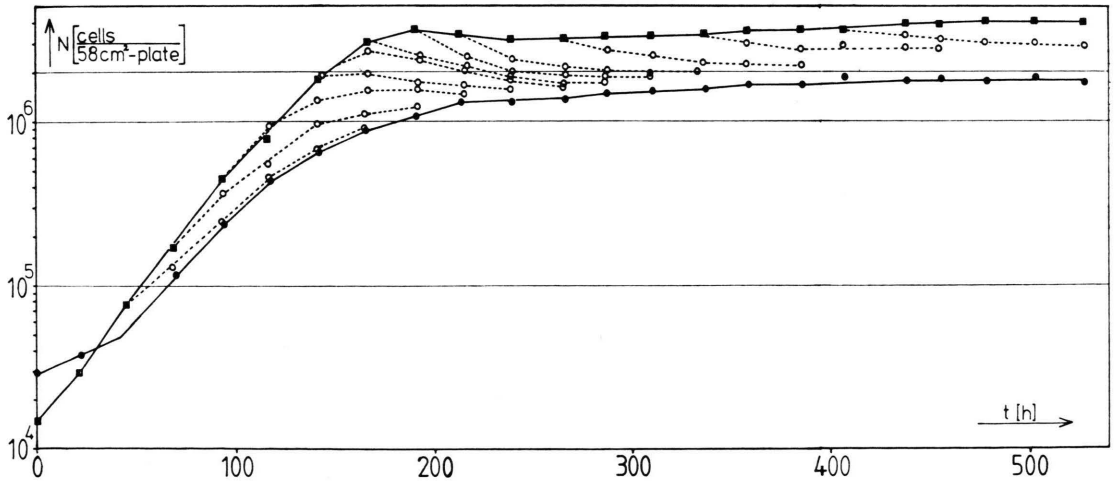

Fig. 1. 3T3 cells were seeded at zero time in 15 ml medium per plate at 2.5% (filled circles) and 10% (filled squares) serum. Population development after serum reduction (10% → 2.5%) is indicated by dashed lines. Serum reductions have been performed at the origins of the dashed lines. All cultures received daily medium renewals (15 ml per plate).

grams that growth retardation per cell is much more abrupt at a serum concentration of 10% than at 2.5%. In Fig. 4, $1/T_d$ is plotted against time for population kinetics after sudden serum reductions as shown in Fig. 1. Population kinetics depends strongly on cell density at time of reduction. At low cell density, decrease of growth rate in dependence on cell density and time is similar in control populations and after a serum reduction (compare first dashed line in Fig. 1 with 2.5% control in Fig. 1 and the solid line with open circles in Fig. 4 with the 2.5% control in Fig. 2). At densities higher than 5×10^5 cells per plate (58 cm²) the decrease of growth rate following serum reductions is much steeper, and at densities higher than the stationary density at 2.5% serum negative growth rates are observed after serum reductions, indicating cell loss caused by cell death as judged microscopically. The rate of cell loss following serum reduction is maximum around the time of saturation of the cultures under 10% serum and decreases again the older the stationary population grows. Thus, very different population behaviour is observed in 3T3 cell cultures at 2.5% serum depending on cell density and on whether a pretreatment with 10% serum has taken place.

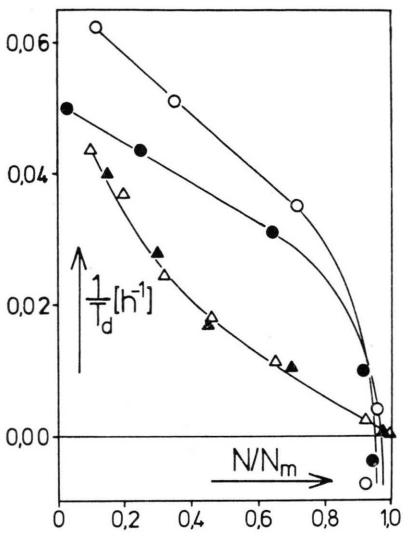

Fig. 3. 3T3 cell population growth rate in dependence on cell density at 2.5% (triangles) and 10% (circles) serum. Abscissa: Normalized cell density N/N_m, where N_m is the maximum cell density reached in an experiment. Filled symbols: data taken from Fig. 1. Open symbols: data taken from growth curves not shown here.

In the following, we describe how these different conditions affect the net interaction between 3T3 and SV40-3T3 cells in mixed cultures. In Fig. 5A, the increase of total cell number (filled circles) has been recorded in dependence on time in 3T3 cell cultures at 2.5% serum onto which SV40-3T3 cells were seeded at the times indicated by arrows. In each case, the ratio of SV40-3T3 cell number to 3T3 cell number was 1 : 3 at the seeding time of the SV40-3T3 cells. The approximate number of SV40-3T3 cells in the mixed population (N_{MSV}) is given by the difference between total cell counts in mixed (filled circles) and pure 3T3 control (open circles) cultures and is plotted also (crosses). Fig. 5B shows N_{MSV} versus time in cultures in which the 3T3 cells experienced a serum reduction from 10% to 2.5% at the time of seeding of the SV40-3T3 cells,

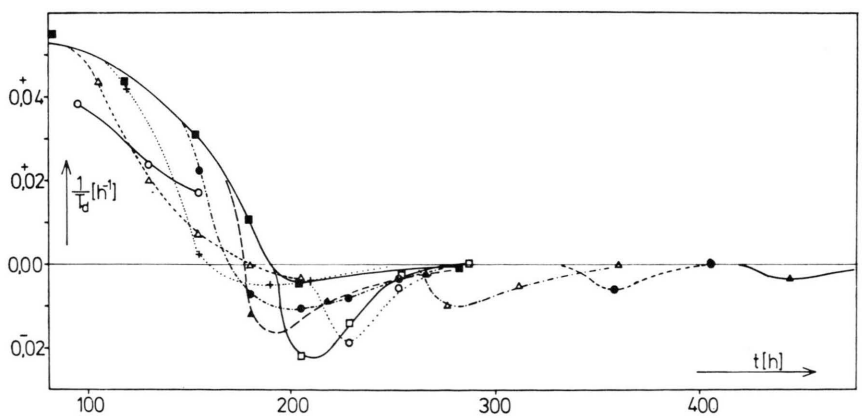

Fig. 4. Rate of cell number change in 3T3 cell populations after serum reductions (10% → 2.5%) at various cell densities and times after seeding in dependence on time. Negative values of T_d indicate cell loss. Filled squares: 10% serum (taken from Fig. 3). Lines connect data from one population development each after serum reduction. Data taken from Fig. 1 as described in the text. For clarity data from the second serum reduction in Fig. 1 have been omitted here.

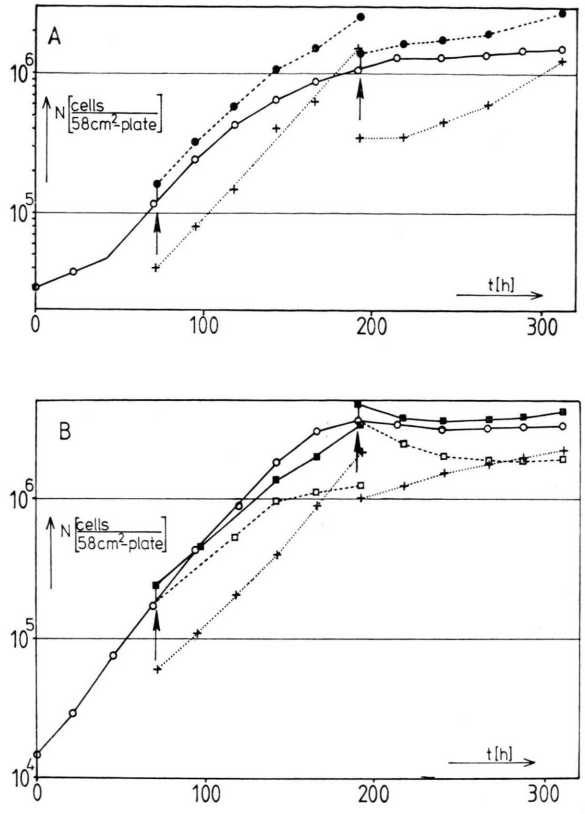

Fig. 5. Population development in mixed cultures of 3T3 and SV40-3T3 cells. At arrows SV40-3T3 cells were seeded in medium containing 2.5% serum onto 3T3 cell populations, which were grown under 2.5% serum (A) or 10% serum (B). The ratio of SV40-3T3 cells to 3T3 cells was about 1:3 at each arrow. A. Open circles: 3T3 control at 2.5% serum; filled circles: mixed 3T3/SV40-3T3 population; crosses: difference. B. Open circles: 3T3 cells at 10% serum; open squares: 3T3 control after serum reduction (10% → 2.5%); filled squares: mixed 3T3/SV40-3T3 population; crosses: difference. All cultures received medium renewals every day (15 ml per 58 cm² plate).

which themselves did not experience this serum reduction, since they had been cultured at 2.5% serum for weeks before seeding onto the 3T3 cell cultures. In both cases (Figs. 5A and B) a marked decrease of R_{MSV} can be observed in dependence on 3T3 cell density. Seedings of SV40-3T3 cells onto 3T3 cell populations and in parallel onto control plates without 3T3 cells have been performed throughout the whole experiment depicted in Fig. 1, and growth has been followed for 5 days each. As a measure of net interaction between 3T3 and SV40-3T3 cells in the mixed populations the quotient R_{MSV}/R_{CSV} has been plotted against time in Fig. 6. R_{MSV} and R_{CSV} are the average log growth rates of SV40-3T3 cells in mixed and in parallel control cultures (without 3T3 cells) respectively. Regarding Fig. 1 and Fig. 6 the following effffects can be seen: at low 3T3 cell density R_{MSV} is larger by a factor of $\approx$ 1.8 than R_{CSV}, which is consistent with a net stimulating effect in the mixed 3T3/SV40-3T3 cell populations. With increasing 3T3 cell density the development of a strong net growth-inhibiting effect is observed, which rises roughly in synchrony with growth reduction in pure 3T3 control cultures. It reaches a plateau where R_{MSV} is smaller by factors of 9 or 14 than R_{CSV} depending on whether the serum concentration has been kept constant (2.5%) or has been reduced (10% → 2.5%) at the time of seeding of the SV40-3T3 cells.

Unfortunately, the number of dead cells in cultures after serum reduction is difficult to evaluate separately, since they disintegrate during removal from the culture dish by pipetting. However, estimates from microscopic examination of the culture

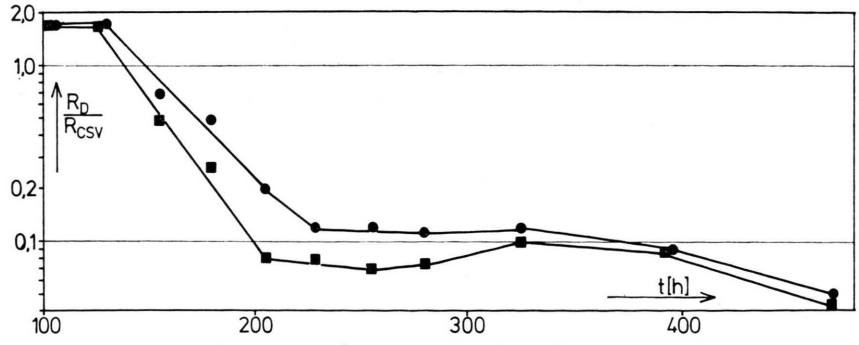

Fig. 6. Relative growth rate R_{MSV}/R_{CSV} of SV40-3T3 cells in mixed cultures in dependence on time. Data are taken from the experiment, the controls of which are shown in Fig. 1, as described in the text. Squares: data from populations following a serum reduction (10% → 2.5%). Circles: data from populations at 2.5% serum without reduction.

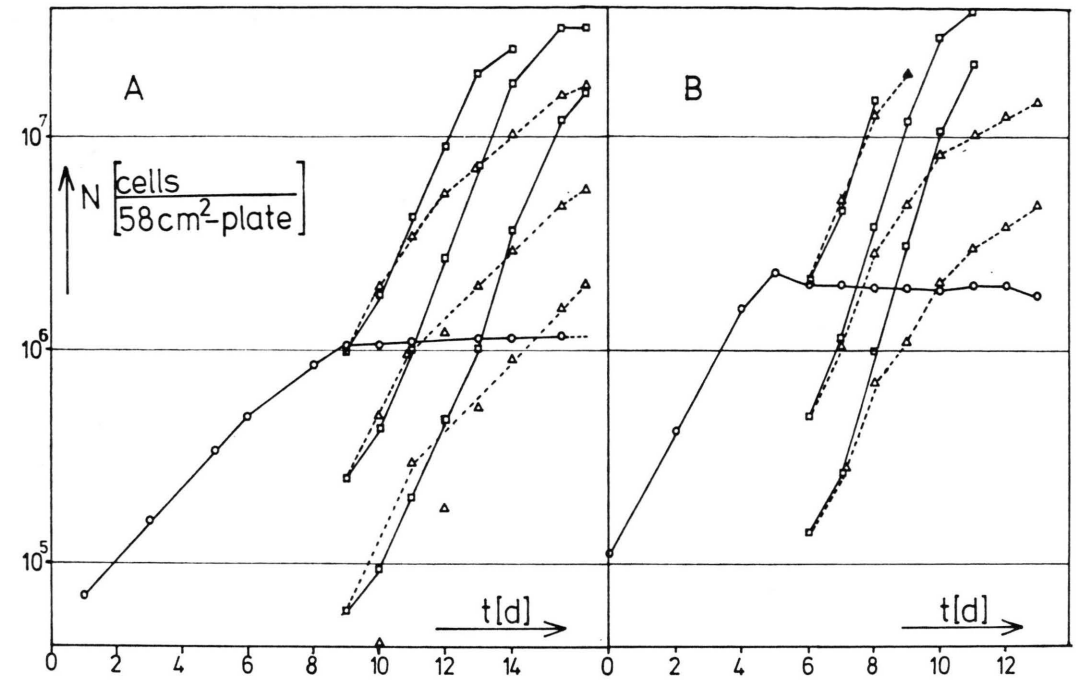

Fig. 7. Dependence of growth-inhibiting interaction in mixed cultures on the ratio of 3T3 to SV40-3T3 cell numbers. At zero time 3T3 cells (open circles) were seeded at 2.5% serum (A) and 10% serum (B). At saturation time SV40-3T3 cells were seeded at cell number ratios of 1:1, 1:4 and 1:16 (SV40-3T3:3T3) onto 3T3 cell cultures (triangles, dashed lines) and onto control plates without 3T3 cells (squares, solid lines). All cultures received medium renewals every second day (10 ml per 58 cm² plate).

plates revealed that the number of dead cells was consistently smaller by 30% to 50% in mixed cultures as compared to pure 3T3 control cultures. Thus, the differences N_{MSV} are maximum values of SV40-3T3 cell numbers in mixed cultures and very probably the growth inhibiting effect on SV40-3T3 cells in such cultures is even stronger than suggested by Fig. 6. Between 250 and 500 h after seeding of the 3T3 cells, death rates following serum reductions in homogeneous 3T3 cultures decrease, and cell density does not appear to approach the value of the control cultures under 2.5% serum (Fig. 1).

Concomitantly, morphologic alterations of the 3T3 cells are observed: stellate cell shapes predominate in these old populations. The viability of these cells seems unaffected as jugded from growth after reseeding. A further decrease of R_{MSV}/R_{CSV} occurs in these old cultures as compared with younger ones (Fig. 6).

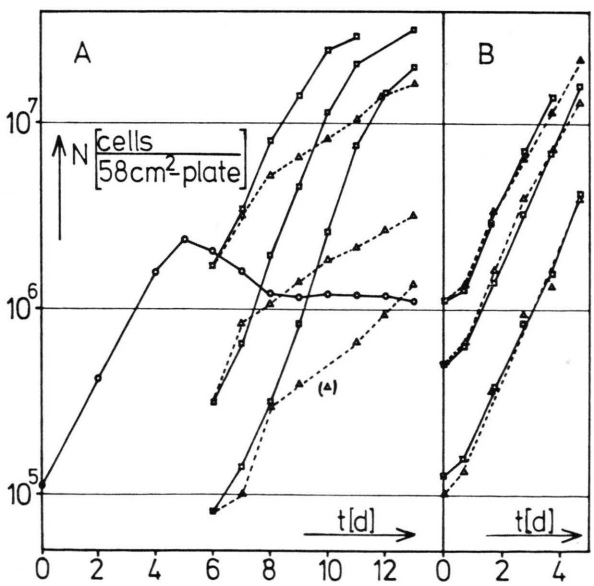

Fig. 8. A. Same experiment as shown in Fig. 7 B, but with serum reduction (10% → 2.5%) for 3T3 cells on day 6 concomittantly with SV40-3T3 cell seeding. B. Growth of homogeneous SV40-3T3 cell populations under fresh medium (squares, solid lines) and under medium conditioned by 3T3 cells during two days. Conditioned medium was taken from 3T3 cell cultures on days 8, 10, and 12 (Fig. 8 A) for seeding at zero time and medium renewals on days 2 and 4, respectively.

Dependence of SV40-3T3 cell multiplication in mixed populations on the ratio of cell numbers of transformed and normal 3T3 cells

In Figs. 7 and 8a the growth of SV40-3T3 cells seeded onto stationary 3T3 cell cultures in proportions of 1 : 16, 1 : 4 and 1 : 1 (SV40-3T3 : 3T3), which is indicated by the dashed lines, is compared with the growth of the same seeding numbers of SV40-3T3 cells in control plates without 3T3 cells (open squares, solid lines). The experiment has been performed under 3 different conditions: at 2.5% serum (Fig. 7A), at 10% serum (Fig. 7B) and with a serum reduction for 3T3 cells from 10% to 2.5% at the time of seeding of the SV40-3T3 cells (Fig. 8A). Under all three schedules a strong reduction of SV40-3T3 cell growth in the mixed cultures is observed as compared to growth in SV40-3T3 control cultures. Surprisingly, this reduction of growth rate seems largely independent of the ratio of SV40-3T3 to 3T3 cells. Differences of SV40-3T3 growth between the three different schedules rest mainly in the time which passes after SV40-3T3 cell seeding until a strong reduction of growth rate becomes visible. Under 10% serum (Fig. 7B) this time is about 3 to 4 days whereas it is only about 2 days under the conditions of Fig. 7A (2.5% serum) and Fig. 8A (10% → 2.5% serum).

In Fig. 8B, a control experiment is shown. SV40-3T3 cells have been grown from three different seeding densities under medium (2.5% serum), which had been conditioned by 3T3 control cultures during the period of time when pronounced inhibition is observed in Fig. 8A. SV40-3T3 population growth under such conditioned medium (dashed lines) is not reduced as compared to growth under fresh unconditioned medium (solid lines in Fig. 8B).

Dependence of SV40-3T3 cell multiplication in mixed populations on pretreatment of SV40-3T3 cells with different trypsin concentrations and EDTA

Variation of the concentration of trypsin used for detachment of SV40-3T3 cells prior to their seeding onto 3T3 cell layers in the range from 1 to 10 mg trypsin per 100 ml and the presence or absence of Mg^{2+} and Ca^{2+} ions during trypsinization did not affect to a measurable extent the growth reduction observed in mixed cultures. Even detachment of SV40-3T3 cells by EDTA (3×10^{-5} m) in the absence of trypsin had no influence on the observed growth kinetics in mixed cultures as compared to these kinetics after trypsin treatment of the SV40-3T3 cells.

Discussion

The present work shows that the rate of total growth of a mixed culture of SV40-3T3 and stationary 3T3 cells is strongly reduced as compared to growth resulting if no interactions take place between the two cell types. This growth reducing interaction is not due to gross depletion of medium components or to other 3T3-growth dependent variations of the culture medium, since the inhibiting effect cannot be transferred from 3T3 cultures to homogeneous SV40-3T3 cultures by transfer of conditioned medium (Fig. 8B). Thus, close cellular proximity seems to be a necessary condition for effective reduction of the SV40-3T3 population growth rate. Similar results have been obtained by Stoker [10, 11] and Stoker *et al.* [14] for growth-inhibiting interactions between BHK21 hamster fibroblasts and their polyoma-virus transformed counterparts. Borek and Sachs [1] described inhibiting interactions between hamster cells transformed by different carcinogens and between normal and transformed hamster cells. No inhibiting interactions could be detected between hamster cells transformed by the same carcinogen. Thus, the inhibiting interactions do display some specificity. This is also obvious in the work of Pontén and Macintyre, who showed that growth-reducing interactions exist between bovine fibroblasts and their polyoma-transformed counterparts, whereas such interactions are not detectable between bovine fibroblasts and their Rous-sarcoma transformed counterparts [7, 8].

In the present work, we show that manipulations (serum reduction) causing an acceleration of density-dependent inhibition of growth and an increase in density-dependent rates of cell death in pure 3T3-cell populations also lead to increased growth-inhibiting interactions between 3T3 and SV40-3T3 cells in mixed populations. These data are strong evidence in favour of a common mechanism at the basis of growth-inhibiting interactions among 3T3 cells and between 3T3 cells and their SV40-transmormed counterparts. Clearly, in no instance total cessation of SV40-3T3 cell multiplication has been observed. However, reception of growth-inhibiting signals, transduction of these signals and the reactions leading to growth reduction seem intact at least in part in these cells. Comparing the interaction

of SV40-3T3 cells with each other and the interaction of SV40-3T3 cells with untransformed 3T3 cells, the conclusion seems unavoidable that the generation of growth-inhibiting signals is strongly disturbed in this transformed cell. Thus, one of the most important questions in this field concerns the nature of the growth-inhibiting signal(s). Taking into account the result that 3T3 cell growth causes depletion of substances necessary for 3T3 cell growth but not SV40-3T3 cell growth and vice versa [9], the most probable explanation of the present results is that the observed reduction of SV40-3T3 cell growth in co-cultures with 3T3 cells is due to inhibiting principles generated by the 3T3 cells. Whether these principles involve diffusion-controlled processes in the extracellular space (probably occurring only in a very restricted micro-environment of the cell) [12] or not [21] is still an open question.

Acknowledgement

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