[CANCER RESEARCH 36, 3126-3130, September 1976]

Spontaneous Cell Loss during Growth of Postconfluent Primary Cultures from Mammary Adenocarcinomas[1]

Howard L. Hosick

Department of Zoology, Washington State University, Pullman, Washington 99163

SUMMARY

Growth properties of cells cultured from primary mammary tumors of C3H mice have been analyzed. Cells were seeded at 2 different densities (1×10^5 and 5×10^5/sq cm) and were supported with a culture fluid containing 10% fetal calf serum and 5 μg insulin per ml. Mitosis continued after confluence was achieved, but cells did not accumulate in the monolayer; rather, certain cells were released into the culture fluid. Very few cells detached in this way from subconfluent cultures. Released cells multiplied vigorously if replated. The release of these cells was strongly depressed by adrenal steroids, but other manipulations of culture conditions (hormones, culture substratum) influenced the release process much less. Analyses of release kinetics and observations of detachment with the scanning electron microscope suggested that tumor cells that became spheroid (including mitotic cells), and hence partly detached from the culture dish, were unable to reflatten into the monolayer because neighboring nonmitotic cells had spread onto the vacated culture surface. Eventually, such rounded cells apparently lost altogether their attachment to the culture dish. The release process may be related to the "critical phase" transition and to the sarcomatous transformation observed in long-term cultures from mouse epithelial tumors. The event could also reflect the tendency *in vivo* for cells of mammary tumors to slough into the lymphatics and blood vessels.

INTRODUCTION

A previous study on mammary adenocarcinoma cells (9) suggested that they stop accumulating after a very limited period of growth in primary culture. This was an unexpected finding which is inconsistent with the usual observation that transformed cells continue to divide and consequently reach very high population densities in culture. The study thus invited a more critical analysis of mammary tumor cell replication in primary cultures.

A few previous observations of transformed cells in culture did provide clues to the nature of the inconsistency. For example, "flat revertant" lines of SV40-transformed 3T3 cells have been described in which cells continue to replicate after confluence, but there is no net cell accumulation in the monolayer (20). The excess cells apparently come loose from the monolayer. Tumor cells are known to be less adherent than are normal cells (28), and dividing cells are particularly nonadherent to substrata in culture (24). Such observations have led me to investigate the shedding of cells from mammary tumor monolayers. Viable cells are, in fact, released into culture fluid at a substantial rate, and this release is a significant factor in the growth pattern.

MATERIALS AND METHODS

Cells were from spontaneous mammary adenocarcinomas of C3H mice. Tumors, 1 to 2 cm in diameter, were removed from mice aseptically, minced, and disassociated with trypsin and EDTA as described elsewhere (10). Resulting cells were seeded into Falcon tissue culture dishes (60 mm in diameter) at densities of 1 and 5×10^5 cells/sq cm substrate area, and incubated at 37° in an atmosphere of 10% CO_2 in air. Standard medium for cell growth was Dulbecco's modified Eagle's medium (5 ml/dish), supplemented with fetal calf serum (10%), bovine insulin (5 μg/ml), and fresh L-glutamine (2 mM). These cultures were confluent in 2 to 6 days, depending on seeding density.

On Day 3 postseeding, spent medium was removed, cultures were rinsed vigorously with warm 0.9% NaCl solution, and fresh medium was added. Thereafter, medium was harvested as gently as possible at 3-day intervals and saved. Each plate was rinsed once with 2 ml prewarmed 0.9% NaCl solution, also saved. Fresh medium was added and plates were reincubated. The harvested medium and respective rinse were combined, and cells were removed centrifugally (5 min $\times$ 100 *g*). Cells were resuspended and stained with trypan blue, and dead and viable cells were enumerated with a hemocytometer, essentially by the method described by Phillips (14). At least 4 cultures were used for each variable at each time point.

For scanning electron microscopy, cells were grown on glass coverslips. (Growth patterns are very similar on glass and tissue-culture plastic substrates.) Rinsed monolayers were fixed in glutaraldehyde, dehydrated in alcohol, and dried by the critical-point method, all essentially as done by Paweletz and Schroeter (13).

All information presented is representative of 3 or more independent experiments in which similar results were obtained.

RESULTS

Chart 1 shows the data obtained when medium was gently removed from cultures and detached viable cells were

[1] Supported by NIH Grant CA-16392 and by funds provided for biological and medical research by State of Washington Initiative Measure 171.

Received January 26, 1976; accepted May 21, 1976.

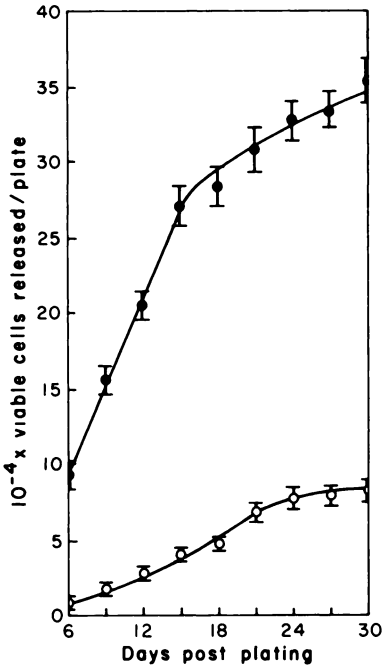


Chart 1. Counts of viable cells recovered from culture fluid. Cells from the same suspension were seeded at densities of 5 × 10^5/sq cm (●) and 1 × 10^5/sq cm (○). Floating cells were harvested and counted from each of 16 cultures on Days 6 to 30. *Vertical bars*, ±S.E. Multiply by 10^4 to obtain correct value.

counted. The bulk of the viable cells was released during the 1st 3 weeks; the rate of detachment declined progressively as culture time increased.[2] Cells were harvested from some cultures for over 18 weeks, and still at least 70% of all the viable released cells were recovered during the 1st 3 weeks. "Nonviable" cells (those that stained with trypan blue) were usually released at a rather low and constant rate (1000 to 3000/culture/day) throughout the life of a culture.

By Day 30, about 5 times more cells had detached from cultures seeded at high density than from cultures seeded at low density (Chart 1). The monolayer phase of cultures seeded at high density was more crowded, but contained only about twice as many cells (9), rather than 5 times as many. Hence, a larger proportion of the monolayer cells was released from the more crowded cultures.

Moreover, in cultures initiated with the lower cell number (1 × 10^5/sq cm), there was usually a lag period of several days before many viable cells were recovered from the medium. This lag could be seen clearly when released-cell counts were graphed as cumulative percentage of the total released during 30 days (Chart 2). A larger proportion of the total was recovered at early harvests from crowded than from sparser cultures. This discrepancy was probably not related to the rates at which cells traversed the cell cycle, since the percentages of cells labeled with [^{3}H]thymidine and undergoing mitosis were similar at these 2 densities during the early days of culture (5). Rather, the lag time in the lower density cultures (seeded at 1 × 10^5 cells/sq cm) corresponded well with the several extra days of growth needed before these cultures became confluent (5). That is to say, very few cells were released from subconfluent cultures. This phenomenon was confirmed by observing cultures seeded at an even lower density (1 × 10^4 cells/sq cm), in which confluency was never attained (9). Hardly any cells were released into the medium throughout the life-span of such subconfluent cultures (fewer than 500 viable cells per culture per day).

Cells released into culture fluid were always spherical, whereas cells in the monolayer were flattened. Cells in the process of detaching might be expected to have shapes intermediate between these extremes. A search was conducted for such intermediate cells. In young, freshly fed monolayers, many rounded, refractile, but attached cells resided in the monolayer lawn (Fig. 1). These were much rarer in older cultures (*i.e.*, after Day 30). Several such rounded cells were inspected more closely with the scanning electron microscope (Fig. 2). They had blebbed and ruffled surfaces, and a few small filopodia extended from them. Such surface features characterize cells entering mitosis (13, 16, 18). Thus, it seems likely that the cells released from the monolayer had rounded up in preparation for mitosis, although this cannot be proven on the basis of such morphological evidence alone. Notice also, however, that the rounded cells essentially sat atop a monolayer of flattened epithelium, with no bare culture dish in evidence. Flattened neighboring cells spread in beneath the rounded cells.

Clumps of cells were not seen in the medium, and cells that were obviously dividing (*i.e.*, dumbbell shaped) were rarely seen. Thus, the floating cells seemed not to replicate in suspension. Released cells might be in the

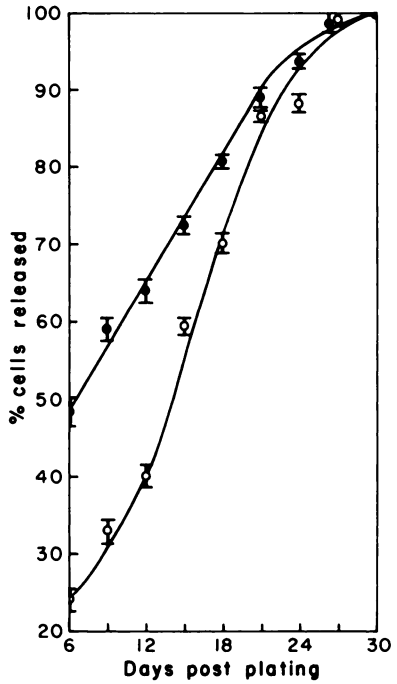


Chart 2. Rate of release at 2 different seeding densities. Cells from the same suspension were seeded at densities of 5 × 10^5/sq cm (●) and 1 × 10^5/sq cm (○). Floating cells were harvested and counted from Days 6 to 30. Total cells released by Day 30 at each density = 100% at that density. *Vertical bars*, ±*S.E.*

[2] In 7 recent experiments using 7 different spontaneous tumors, from 20 to 62% of all floating cells released in 1 month from high-density cultures were already released by Day 6 (average, 34%).

"agonal dying phase" (4) and incapable of dividing, or they may simply be incapable of dividing in suspension. To test further the viability and mitotic potency of the floating cells, they were replated into new culture dishes. Plating efficiency was similar to that in the original cultures. Fig. 3 shows a typical epithelial island that developed from a single replated floating cell. Clearly, this cell was viable and able to multiply when placed in the appropriate environment. Note also that the progeny of this cell were epithelioid, not fibroblastoid (17, 19), and neither were released cells all lymphocytes.

The rate of release was little affected by the substratum on which the cells were grown (glass, plastic, collagen-coated plastic). If the Ca^{2+} concentration in culture medium was doubled, release was only slightly decreased. (However, no blisters or "domes" (11) formed in cultures grown with excess Ca^{2+}, suggesting that the flattened monolayer cells did adhere more tightly to the substratum.) Glucocorticoid hormones, however, strongly depressed the rate of release of viable cells (Table 1). (The number of nonviable cells was virtually identical in cultures with and without hydrocortisone present.) Prolactin, the mammotrophic hormone, only slightly modified the response to hydrocortisone and had no effect by itself (not shown). Thyroxine, another hormone to which mammary cells are responsive, decreased release only slightly.

DISCUSSION

About half a dozen published papers mention in passing the spontaneous release of cells from postconfluent epithelial monolayers. This is a phenomenon of general significance in epithelioid cultures, but the release process has not been analyzed in detail before. Moreover, all of the previous observations were made on monolayer cell lines [including a line of mammary tumor cells (30)]. The present study using *bona fide* adenocarcinoma cells in primary culture suggests that release from monolayers reflects a property of tumors *in vivo*, and also allows me to propose how and why cells detach.

The microscopic appearance of cultures suggests the following explanation for cell release. Some cells in confluent monolayers loosen their junctions with neighboring cells and decrease their area of contact with the culture substratum; these events typify cultured cells entering mitosis. The vacated substratum is immediately occupied by neighboring epithelial cells, which are extremely plastic and able to spread out rapidly and extensively (5). A rounding cell gradually loosens its grip on the culture dish. Finally, its narrowed connection with the substratum is broken; the cell is then free-floating atop a confluent epithelial monolayer, to which it is unable to adhere (7, 18, 27). Eventually, a substantial proportion of the mitotic cells is lost and the rate of release declines. The discrepancy in behavior between confluent and subconfluent cultures lends credence to this model of cell release. In subconfluent cultures, postmitotic cells are not deprived of a surface to which they can adhere, but rather are able to reflatten onto the surrounding bare culture dish.

Loss of cells is a major factor determining rate of growth of C3H mammary tumors. The calculations of Steel (23) indicate that the cell loss factor is 69%. Similar losses are noted also in a wide range of human tumors (4, 22). Butler and Gullino (3) have directly measured the number of cells shed by a transplantable mammary tumor (rat) into efferent vessels; the number is 3.2×10^6 cells per g tumor per day. It is clear that sloughing of cells from the periphery of a tumor is a biologically important trait. The mechanism underlying cell release from tumors is unknown, but a propensity to detach from neighbors is evident also in the cells when cultured. It is interesting to measure responses in culture to treatments that affect detached cells in animals. The corticoids, for example, promote metastasis of experimental adenocarcinomas (1, 6, 31). Cell release from culture monolayers was decreased by a corticoid, hydrocortisone (Table 1). This is consistent with the fact that glucocorticoid-treated cells adhere more tightly to the substratum in culture (2, 21). Besides their other effects, glucocorticoids may likewise cause tumor cells to adhere more tightly *in vivo*. This could be shown by counting the cells released from mammary tumors into efferent vessels before and after corticoid treatment.

In mouse mammary tumors *in vivo*, less than one-half of the cells are engaged in the cell cycle (12), and furthermore the noncycling cells are distinctly differentiated ultrastructurally (29). Likewise, only a small proportion of the total cells in a primary monolayer of mammary cells is traversing the cell cycle (5), and the typical monolayer cell possesses features suggestive of differentiation; for example, apical microvilli and occluding junctions (15). The cell populations in primary culture seem to reflect rather accurately the cell populations in the original tumor. The most important population of tumor cells is the growth fraction, and the mitotic cells released from postconfluent monolayer cultures must represent at least part of this population. Unfortunately, these cells are discarded with spent culture medium, and eventually much of the cycling population may be rinsed away from the monolayer, leaving behind the static, differentiated cells. Such depletion of cycling cells is indicated by release kinetics (Chart 1), *i.e.*, the rate of cell release declines as culture time increases. Twentyman *et al.* (25, 26),

Table 1

Effect of hormones on release of cells

Cells were seeded at 5×10^5/sq cm in standard medium. Plates were rinsed and medium was replenished every 3 days. On Day 6, all cells received standard medium (Condition 1) or standard medium plus: 10 μg hydrocortisone per ml (Condition 2); 10 μg/ml each, hydrocortisone and prolactin (Condition 3); or 10 μg/ml thyroxine (Condition 4). These media were replaced with fresh media of the same compositions every 3 days, and cells floating in spent media were enumerated at each replacement.

Conditions	Σ viable cells released[a] ($\times 10^3$)
1. Standard	102 ± 12[b]
2. +hydrocortisone	34 ± 9
3. +hydrocortisone and prolactin	49 ± 1
4. +thyroxine	86 ± 8

[a] Σ = dye-excluding cells harvested from Day 9 through Day 36 postseeding. Multiply by 10^3 to give correct value.
[b] Mean ± S.E.

studying a line of alveolar tumor cells, also found that the rate of cell loss changed during postconfluent growth, and their observations also suggest to me the selective release of a replicating cell population. Analyses of tumor cell growth in culture should take this released population into account.

Indeed, such loss of cells may help account for several puzzling phenomena which occur as neoplastic mouse epithelia are cultured for long periods of time, so as to establish cell lines. Early-passage cultures go through a postconfluence "dormant period" lasting 1 to 3 weeks, during which few cells accumulate in the monolayer. Dormancy is noted, for example, in secondary and tertiary cultures from BALB/cfC3H mouse mammary tumors (30). This dormant period coincides well with the time during which cell loss to medium is most vigorous. The dormancy may, therefore, be apparent only if new cells are lost from the monolayer. Whether the monolayer cells which eventually proliferate to produce a cell line represent a population distinguishable from the population lost to the medium remains to be demonstrated. It is known, however, that long-term cultures of epithelial tumor cells, including those from mouse mammary adenocarcinomas, tend to transform into cells that look like fibroblasts in culture. These produce sarcoma-type tumors when replanted into mice (19). If the original epithelial cells are indeed lost selectively from cultures, as the results here reported suggest, then a population of cells of another type might eventually dominate older cultures. Such a population could be: (*a*) present only in small numbers in primary cultures, (*b*) derived from cells of the original type which become altered during culture, (*c*) traversing the cell cycle very slowly in early-passage cultures, (*d*) at a competitive disadvantage when cocultured with epithelial tumor cells, etc. This venerable problem needs to be reconsidered.

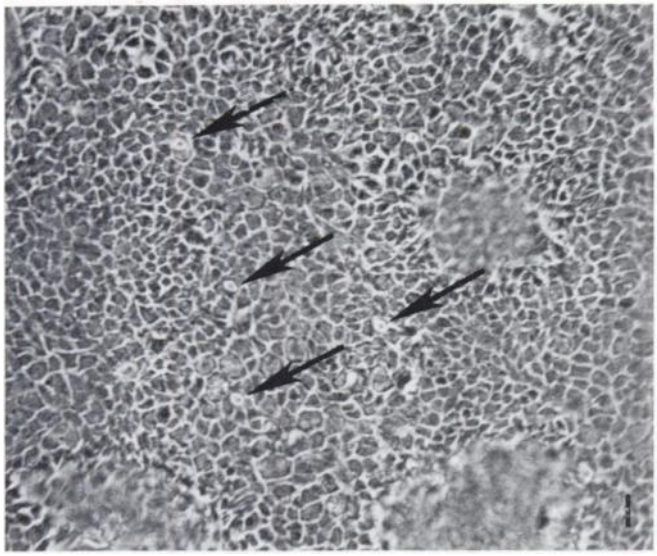
In sum, I concur with several other culture biologists (*e.g.*, Refs. 8, 26) who have pointed out recently that postconfluent cultures of tumor cells hold promise as model systems in which tumor cell properties can be accurately and meaningfully analyzed. It is now clear that postconfluent monolayer cultures of mammary adenocarcinoma cells must be thought of as 3-dimensional systems, with loss of cells to culture medium a potentially significant factor in the growth pattern.

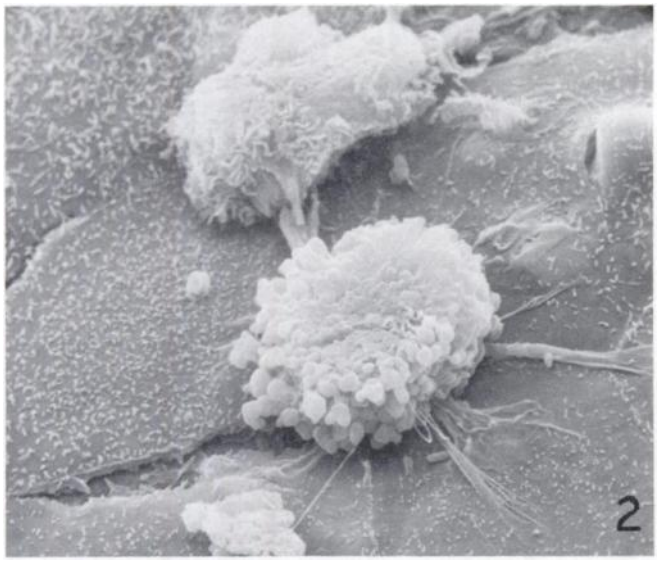
ACKNOWLEDGMENTS

Electron microscopy was performed with the assistance of the Electron Microscope Center, Washington State University. S. Gwynne merits my thanks for technical assistance. I wish to especially commend the careful help of M. Wirth, now deceased.

REFERENCES

1. Agosin, M., Christen, R., Badinez, O., Gasic, G., Neghme, A., Pizarro, O., and Japra, A. Cortisone-Induced Metastases of Adenocarcinoma in Mice. Proc. Soc. Exptl. Biol. Med., *80:* 128-131, 1952.
2. Ballard, P. L., and Tomkins, G. M. Hormone Induced Modification of the Cell Surface. Nature, *224:* 344-345, 1969.
3. Butler, T. P., and Gullino, P. M. Quantitation of Cell Shedding into Efferent Blood of Mammary Adenocarcinoma. Cancer Res., *35:* 512-516, 1975.
4. Cooper, E. H. The Biology of Cell Death in Tumours. Cell Tissue Kinet., *6:* 87-95, 1973.
5. Das, N. K., Hosick, H. L., and Nandi, S. Influence of Seeding Density on Multicellular Organization and Nuclear Events in Cultures of Normal and Neoplastic Mouse Mammary Epithelium. J. Natl. Cancer Inst., *52:* 849-861, 1974.
6. DeBrabander, M., Aerts, F., and Borgers, M. The Influence of a Glucocorticoid on the Lodgement and Development in the Lungs of Intravenously Injected Tumour Cells. European J. Cancer, *10:* 751-755, 1974.
7. Elsdale, T., and Bard, J. Cellular Interactions in Morphogenesis of Epithelial Mesenchynal Systems. J. Cell Biol., *63:* 343-349, 1974.
8. Hahn, G. M., and Little, J. B. Plateau Phase Culture of Mammalian Cells: an *in Vitro* Model for Human Cancer. Current Topics Radiation Res., *8:* 39-83, 1972.
9. Hosick, H. L. A Note on Growth Patterns of Epithelial Tumor Cells in Primary Culture. Cancer Res., *34:* 259-261, 1974.
10. Hosick, H. L., and Nandi, S. Plating and Maintenance of Epithelial Tumor Cells in Primary Culture: Interacting Roles of Serum and Insulin. Exptl. Cell Res., *84:* 419-425, 1974.
11. Hosick, H. L., and Nandi, S. Preliminary Survey of Hormonal Influences on Multicellular Architecture in Primary Cultures of Mammary Carcinoma Cells. J. Natl. Cancer Inst., *52:* 897-902, 1974.
12. Mendelsohn, M. L. Autoradiographic Analysis of Cell Proliferation in Spontaneous Breast Cancer of C3H Mouse. III. The Growth Fraction. J. Natl. Cancer Inst., *28:* 1015-1029, 1962.
13. Paweletz, N., and Schroeter, D. Scanning Electron Microscopic Observations on Cells Grown *in Vitro*. II. HeLa Cells in Mitosis. Cytobiologie, *8:* 238-246, 1974.
14. Phillips, H. J. Dye Exclusion Tests for Cell Viability. *In:* P. F. Kruse, Jr., and M. K. Patterson, Jr. (eds.), Tissue Culture Methods and Applications, pp. 406-408. New York: Academic Press, Inc., 1973.
15. Pickett, P. B., Pitelka, D. R., Hamamoto, S. T., and Misfeldt, D. S. Occluding Junctions and Cell Behavior in Primary Cultures of Normal and Neoplastic Mammary-Gland Cells. J. Cell Biol. *66:* 316-332, 1975.
16. Porter, K., Prescott, D., and Frye, J. Changes in Surface Morphology of Chinese Hamster Ovary Cells during the Cell Cycle. J. Cell Biol., *57:* 815-836, 1973.
17. Prop, F. J. A. Is the Upper Cell Surface Unable to Support Active Cell Movement in Culture? J. Cell Biol., *66:* 215-219, 1975.
18. Rajarman, R., Rounds, D. E., Yen, S. P. E. S., and Renbaum, A. A Scanning Electron Microscope Study of Cell Adhesion and Spreading *in Vitro*. Exptl. Cell Res., *88:* 327-339, 1974.
19. Sanford, K. K., Dunn, T. B., Westfall, B. B., Covalesky, A. B., Dupree, L. T., and Earle, W. R. Sarcomatous Change and Maintenance of Differentiation in Long-Term Cultures of Mouse Mammary Carcinoma. J. Natl. Cancer Inst., *26:* 1139-1183, 1961.
20. Scher, C. D., and Nelson-Rees, W. A. Direct Isolation and Characterization of "Flat" SV40-Transformed Cells. Nature, *233:* 263-265, 1971.
21. Shields, R., and Pollock, K. The Adhesion of BHK and PyBHK Cells to the Substratum. Cell, *3:* 31-38, 1974.
22. Steel, G. G. Cell Loss as a Factor in the Growth Rate of Human Tumors. European J. Cancer, *3:* 381-387, 1967.
23. Steel, G. G. Cell Loss from Experimental Tumours. Cell Tissue Kinet., *1:* 193-207, 1968.
24. Terasima, T., and Tolmach, L. J. Growth and Nucleic Acid Synthesis in Synchronously Dividing Populations of HeLa Cells. Exptl. Cell Res., *30:* 344-362, 1963.
25. Twentyman, P. R., and Bleehen, N. M. Changes in Sensitivity to Radiation and to Bleomycin Occurring During the Life History of Monolayer Cultures of a Mouse Tumor Cell Line. Brit. J. Cancer, *31:* 68-74, 1975.
26. Twentyman, P. R., Watson, J. V., Bleehen, N. M., and Rowles, P. M. Changes in Cell Proliferation Kinetics Occurring during the Life History of Monolayer Cultures of a Mouse Tumour Cell Line. Cell Tissue Kinet., *8:* 41-50, 1975.
27. Vasiliev, J. M., Gelfand, I. M., Domnina, L. V., Zacharova, O. S., and Ljubimov, A. V. Contact Inhibition of Phagocytosis in Epithelial Sheets: Alterations of Cell Surface Properties Induced by Cell-Cell Contacts. Proc. Natl. Acad. Sci. U.S., *72:* 719-722, 1975.
28. Weiss, L. The Cell Periphery, Metastasis, and Other Contact Phenomena. New York: John Wiley & Sons, 1967.
29. Wylie, V. C., Nakane, P. K., and Pierce, G. B. Degrees of Differentiation in Nonproliferating Cells of Mammary Carcinoma. Differentiation, *1:* 11-20, 1973.
30. Yagi, M. J. Cultivation and Characterization of BALB/cfC3H Mammary Tumor Cell Lines. J. Natl. Cancer Inst., *51:* 1849-1860, 1973.
31. Yuhas, J. M., and Pazmiño, N. H. Inhibition of Subcutaneously Growing Line 1 Carcinomas Due to Metastatic Spread. Cancer Res., *34:* 2005-2010, 1974.





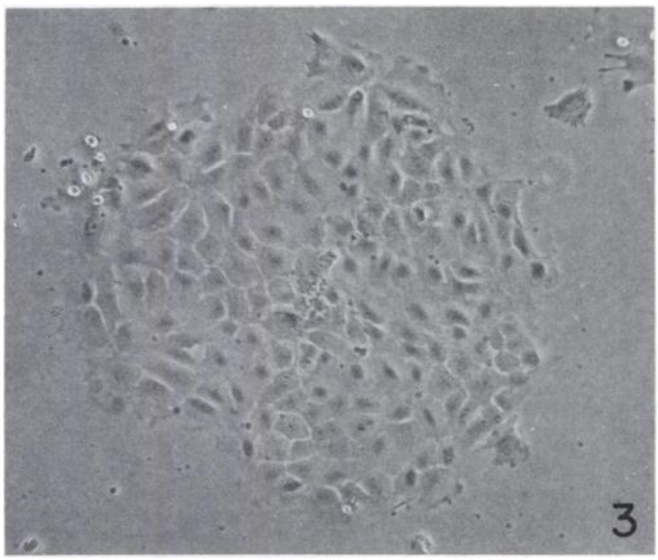


Fig. 1. Morphology of rounded cells in postconfluent culture. Cells were seeded at 5 × 10^5/sq cm in standard medium. Phase-contrast photo (× 100) of living culture, Day 12 postseeding. Note scattering of highly refractile, rounded cells (*arrows*). [Out-of-focus areas are secretory blisters that indicate the healthiness of the culture (5)].

Fig. 2. Scanning electron micrograph of 2 rounded cells. A culture like that in Fig. 1 was fixed and area containing these rounded cells was marked. This area of the substrate was observed with the scanning electron microscope. The surface characteristics of these 2 cells appear to be different. × 2400.

Fig. 3. An island of epithelial cells that developed from a single floating cell. Cells were seeded at 5 × 10^5/sq cm in standard medium. Medium was changed as usual on Days 3 and 6; on Day 9, cells were harvested from medium and replated in standard medium in new dishes. Phase contrast of living culture. × 100.