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Properties of Enucleated Cells: II. Characteristic Overlapping of Transformed Cells Is Reestablished by Enucleates§

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Summary

Many normal cells are restricted in movement over each other's surfaces. We have quantitatively examined this restriction in the established mouse cell line 3T3, in a polyoma virus-transformed subline Py3T3, and in enucleated cytoplasms prepared from these lines by cytochalasin B treatment.

Overlapping of trypsinized, replated cells was quantitated by phase contrast and scanning electron microscopy. Cells of the normal mouse line 3T3 did not overlap one another. Cells of Py3T3 overlapped one another extensively. Trypsinized, replated 3T3 enucleates did not overlap one another, whereas replated enucleates of Py3T3 displayed many overlaps.

Introduction

Cells of the 3T3 line derived from a Swiss mouse embryo (Todaro and Green, 1963) cease to move upon contact with one another (Castor, 1971). Apparently movement is in some way a prerequisite to continued cell division in this line, for the eventual result of this contact inhibition of movement (Abercrombie and Heaysman, 1954) is to restrict the maximum number of cells occupying a given area to that number capable of residing side by side as a monolayer. This monolayer density is then maintained indefinitely (Todaro and Green, 1963; Stoker, 1964; Castor, 1971).

Transformed sublines, derived from 3T3 by infection with the tumor viruses polyoma or SV40, have lost this sensitivity to contact. As a result, they overlap one another upon contact, and growth proceeds—even to very high cell densities (Abercrombie, 1962; Todaro, Green, and Goldberg, 1964; Risser and Pollack, 1974).

The nuclei of normal or transformed cells growing on solid substrates can be removed from the cells by centrifugation at 37°C in nutrient medium containing the drug cytochalasin B (Prescott, Myerson, and Wallace, 1972; Poste and Reeve, 1972; Pollack and Goldman, 1973; Goldman and Pollack, 1974). Enucleated cells have been shown to preserve many characteristics of the whole cells from which they are derived. In particular, when enucleated normal cells are trypsinized and replated, the enucleates are able to carry out the processes of attachment, spreading, pinocytosis, cell locomotion, and contact inhibition of movement for at least 10 hr (Goldman, Pollack, and Hopkins, 1973).

In this paper we have replated 3T3, Py3T3, and enucleates of each line in order to determine whether the enucleated cytoplasms of untransformed and transformed cells responded differently to cell–cell contact.

Results

Definition of Cytoplasmic Overlap Index

To compare the social behavior of normal and transformed nucleated cells with their enucleated cytoplasms, we were obliged to assay an event that was expressed within the 12–24 hr lifetime (Wright, 1973) of the enucleates. Overlapping of one transformed cell or enucleate by another does occur within 6 hr after replating at subconfluent densities, so we chose to quantitate this expression of cell–cell interaction for all samples. Both the light microscope and the SEM viewed the cells from above (Figure 1). Extensive interdigitation of cell edges therefore was not resolved by either technique, whereas processes extending over adjacent cells were seen easily. The SEM increased resolution of thin processes.

One source of possible error in interpreting such overlaps is their obvious dependence on cell density. Cells therefore were replated at subconfluent densities, so that the fraction of cells forced to overlap would be small. Then, in order to eliminate any remaining dependence of our measure of overlapping on cell density, we counted the fraction of cells in contact with one or more other cells, as well as the fraction of cells overlapping one or more other cells. The ratio of these two observable numbers is the cytoplasmic overlap index. This number, which ranges from 0 to 1, expresses the fraction of contacting cells that overlap a neighbor (see Table 1).

Observations on Nucleated Cells

After replating, both 3T3 and Py3T3 cells made many contacts (Figure 1). Not all of these contacts resulted in overlapping of cytoplasm. Examples of such nonoverlapping contacts are shown as they appeared in living preparations with phase contrast optics (Figure 1a,b) and in fixed SEM preparations (Figure 1d). Typical overlaps of Py3T3 cells are also shown (Figure 1c,e). Notice the covering of an

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Table 1. Overlapping by Normal and Polyoma-Transformed 3T3 Cells

Line	Technique, Mag	Density cells/cm² × 10⁴	Fraction of Cells*		Cytoplasmic Overlap Index†
			Contacting	Overlapping	
Py3T3	SEM, 1000×	1.3	0.52	0.38	0.72
	Phase, 400×	2.1	0.87	0.063	0.072
		0.32	0.34	0.024	0.071
3T3	SEM, 1000×	0.8	0.86	<0.01	<0.01
	Phase, 400×	0.61	0.57	<0.003	<0.005

*More than 200 cells were examined to determine each fraction.
†Cytoplasmic overlap index = fraction of cells whose cytoplasms overlap at least one other cell/fraction of cells in contact with at least one other cell.

Trypsinized cells were centrifuged and inoculated in drops of 0.005 ml on glass coverslips. One hr following inoculation, coverslips were flooded with medium. Six hr later, coverslips were fixed either in formalin–PBS (for light microscopy) or in glutaraldehyde (for eventual scanning electron microscopy).

Table 2. Overlapping by Enucleates Prepared From Normal and Polyoma-Transformed 3T3 Cells

Line	Technique	Mag	Density cell/cm² × 10⁴	Fraction of Enucleates		Cytoplasmic Overlap Index
				Contacting	Overlapping	
Py3T3	SEM	1000×	2.8	0.77	0.72	0.94
	Phase	400×	2.3	0.51	0.045	0.088
			0.48	0.16	0.025	0.159
3T3	SEM	1000×	1.7	0.92	<0.01	<0.01
	Phase	400×	0.62	0.37	<0.005	<0.014

Enucleates were prepared on plastic coverslips as described in Experimental Procedures. Two hr after centrifugation, when the effects of CB had been reversed, enucleates were trypsinized, centrifuged, and replated in drops of 0.005 ml on glass coverslips. Five to 6 hr later, coverslips were fixed and prepared for phase or scanning electron microscopy.

area of one cell by an area of cytoplasm of another cell.

Contacts and overlapping were examined on sister coverslips by both phase contrast microscopy and scanning electron microscopy, at approximately the same magnification. While qualitatively the results obtained were the same, the increased resolution of the SEM permitted us to detect many overlaps which could not be resolved with the light microscope (Table 1).

As expected, the cytoplasmic overlap index was not dependent upon cell density for the subconfluent drop cultures examined. Overlaps in Py3T3 cultures were more easily seen in the SEM than by light microscopy (Table 1). No overlaps were detected among over 1000 contacted 3T3 cells, even after close SEM examination (Table 1).

Observations on Enucleates

The characteristic different morphologies of 3T3 and Py3T3 were well preserved by their respective enucleated cytoplasms, even after trypsinization and replating. Overlapping by enucleated Py3T3 and 3T3 cells was indistinguishable (Table 2) from overlapping by nucleated cells. No overlapping was seen among contacting replated 3T3 enucleates, but was very common among contacting replated Py3T3 enucleates. Again, the SEM permitted detection of many more overlaps among Py3T3 enucleates, but revealed no overlaps among 3T3 enucleates (Table 2).

Discussion

We have reported on the preservation of normal behavior by trypsinized, replated enucleates in culture (Goldman et al., 1973). Here we show that trypsinized, replated enucleates from normal and transformed cells regenerate the ability to recognize and respond to each other by either overlapping or not overlapping, very much as do the cells from which they are derived. The cytoplasmic overlap index was devised to quantitate the difference in appearance of 3T3 and Py3T3 nucleated as well as enucleated cells.

Two lines of work are opened up by these observations. First, these results and the result of the previous paper (Goldman et al., 1973) encourage us to begin to examine enucleated normal and transformed cells for evidence of the persistence of other characteristic differences between normal and transformed cells (Table 3). Second, it should now be possible to determine whether or not such oncogenic agents as chemical carcinogens and tumor

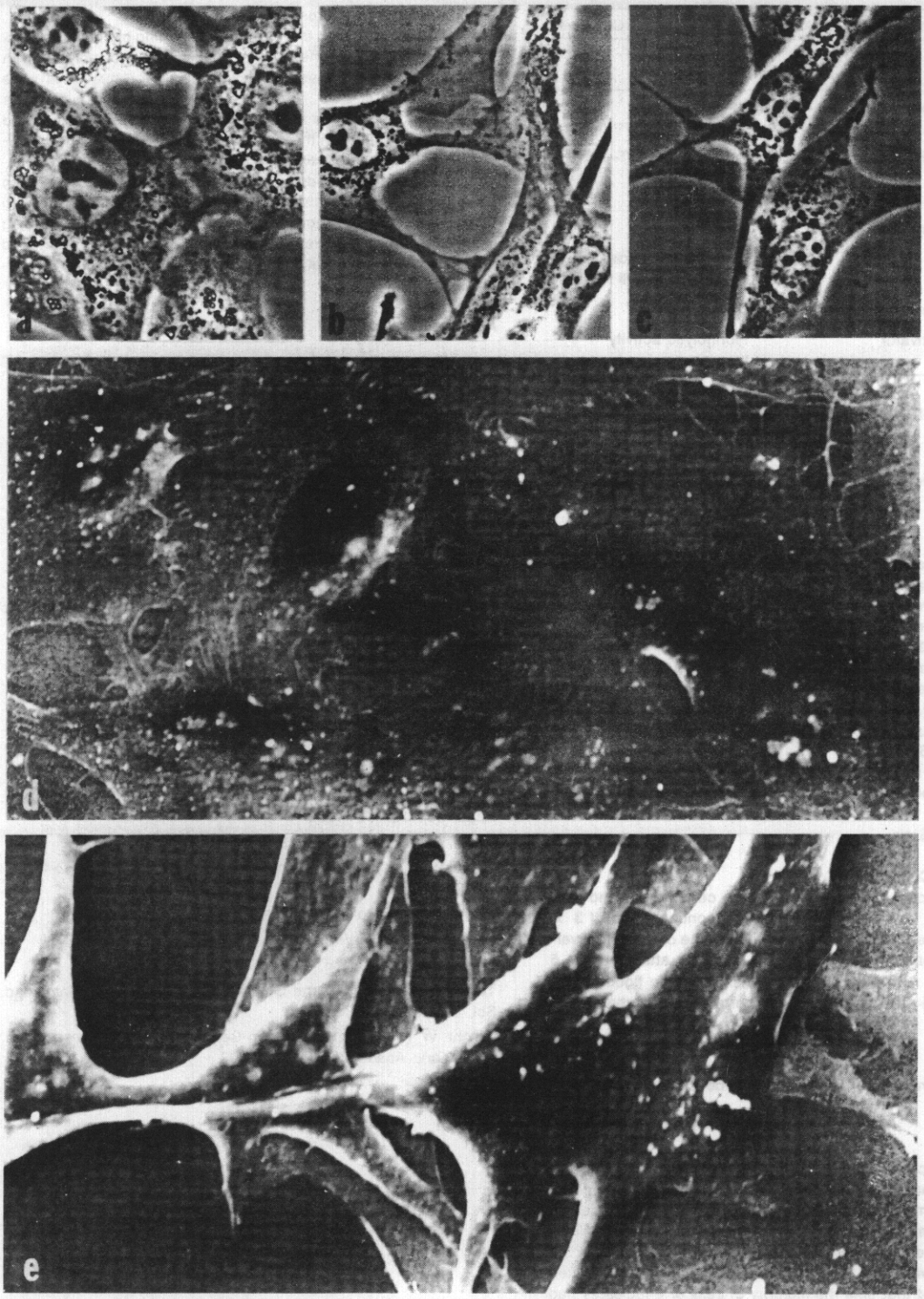

Figure 1. Nucleated Cell Contacts and Overlaps

3T3 and Py3T3 cultures were trypsinized and replated on glass coverslips. They were then either photographed alive with phase contrast optics (a,b,c) or fixed and photographed with the SEM (d,e). 3T3 (a,d) made many contacts, but no overlaps. Py3T3 also made many contacts, and among these, some proceeded to overlap while others did not (b,c,e). Many more overlaps are visible in the SEM preparations, even though final magnifications are approximately equal. Phase contrast micrographs (a,b,c), ×720. Scanning electron micrographs, (d) ×750, (e) ×1500.

Table 3. Differences Between Normal and Transformed Cell Lines

Property	Change Upon Transformation
Contact inhibition of movement	Overlapping Increases
Persistence of virus	Tumor antigen (T-ag) Viral DNA Viral RNA
Agglutinability by lectins	Increases
Morphology	Cells more rounded Surface microvilli increase
Cyclic AMP concentration	Decreases
Secretion of activator of plasmin	Fibrinolytic activity Increases

viruses can increase the overlap index of enucleated 3T3 cells to a detectable value.

Experimental Procedures

Cell Cultures

3T3 is a mouse line derived from Swiss mouse embryo cultures (Todaro and Green, 1963). 3T3 cells have a very low saturation density in 10% calf serum and have a high degree of contact inhibition of cell movement. As a result of these two properties, 3T3 cultures cease increasing in cell number at a monolayer (Todaro and Green, 1963; Risser and Pollack, 1974).

Py3T3 cells are a clone derived from culture of 3T3 infected with the oncogenic papova virus polyoma (Todaro, Green, and Goldberg, 1964). Py3T3 cells do not show contact inhibition and reach a high saturation density (Todaro et al., 1964; Pollack, Green, and Todaro, 1968).

Cultures were maintained at 37°C in three changes/week of Dulbecco's modified Eagle's (DME) medium (Gibco, Grand Island, New York) plus 10% calf serum in a water-saturated atmosphere of 10% CO_2:90% air.

Inoculation of Cells

3T3 or Py3T3 cultures were trypsinized, centrifuged, and suspended in DME plus 10% calf serum at a concentration of 3×10^5 cells/ml. One-half ml was pipetted as a dome onto each coverslip. After incubation for 2 hr at 37°C to permit attachment (1.5×10^5 cells/22 mm coverslip), the coverslips were flooded with 1.5 ml of DME + 10% calf serum and permitted to incubate overnight.

Enucleation

Coverslip cultures were enucleated as described in Goldman et al. (1973).

Replating

Two hr after centrifugation, cells on enucleated and mock-enucleated coverslips were trypsinized, centrifuged, and suspended in small volumes of fresh DME plus 10% calf serum. 5λ aliquots of each suspension were pipetted onto glass coverslips. Cells or enucleates in the drops were permitted to attach for 1 hr at 37°C. The coverslips were then flooded with warm medium and incubated for 6 hr more. At that time the majority of cells or enucleates had fully spread and had begun to move about on the dish. Drops containing cells or enucleates at subconfluent densities were fixed and prepared for either light or scanning electron microscopy. Replated enucleate cultures contained at least 90% enucleated cells.

Scanning Electron Microscopy

Enucleates were fixed at different times following plating onto plastic culture dishes. The medium was poured off and replaced with 1% glutaraldehyde solution. Following this the enucleates were postfixed with 1% OsO_4. Following fixation, the coverslips were passed through a series of alcohols and were finally equilibrated in amyl acetate and run through either a Denton or a Bomar SPC-900 critical point dryer utilizing liquid CO_2 to replace the amyl acetate (for details see Anderson, 1956). The coverslips were then affixed to SEM stubs with silver conductive paint and coated with gold in a Denton high vacuum evaporator equipped with a rotating tilting omnistage. Observations were made with an AMR (Advanced Metal Research Corporation) VTC 1000 scanning electron microscope.

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