LOCOMOTORY BEHAVIOR, CONTACT INHIBITION, AND PATTERN FORMATION OF 3T3 AND POLYOMA VIRUS-TRANSFORMED 3T3 CELLS IN CULTURE

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ABSTRACT

The social behavior of 3T3 cells and their polyoma virus-transformed derivative (Py3T3 cells) was examined by time-lapse cinemicrography in order to determine what factors are responsible for the marked differences in the patterns formed by the two cell lines in culture. Contrary to expectations, both cell types have been found to exhibit contact inhibition of cell locomotion. Therefore, the tendency of 3T3 cells to form monolayers and of Py3T3 cells to form crisscrossed multilayers cannot be explained on the basis of the presence versus the absence of contact inhibition. Moreover, with the exception of cell division control, the social behavior of the two cell types is qualitatively similar. Both exhibit cell underlapping and, after contact between lamellipodia, both show inhibition of locomotory activity and adhesion formation. Neither cell type was observed to migrate over the surface of another cell. The two cell types do show quantitative differences in the frequency of underlapping, the frequency with which contact results in inhibition of locomotion, and the proportion of the cell margin that adheres to the substratum. The increased frequency of Py3T3 underlapping is correlated with the reduced frequency of substratum adhesions, which in turn favors underlapping. On the basis of these observations, it is concluded that the differences in culture patterns are the result of differences in the shapes of the individual cells, such that underlapping, and hence crisscrossing, is favored in Py3T3 cell interactions and discouraged in 3T3 cells.

KEY WORDS contact inhibition · cell movement · pattern formation · transformed cells · invasion

In a series of studies during the 1950s, Abercrombie and his colleagues (6) described a directional restriction of cell locomotion produced by contact between cells, such that the cells fail to crawl over each other's upper surfaces. This phenomenon was termed "contact inhibition" of cell locomotion and was proposed to explain monolayering, i.e., the tendency of fibroblastic cells to remain in a single layer in tissue culture (5). Cells derived from sarcomas, on the other hand, were found to show a reduced amount of contact inhibition and to invade populations of normal fibroblasts (7) by crawling over the surfaces of the fibroblast cells (4). On the basis of these findings, it was proposed that a loss or decrease in contact inhibition might

be responsible for the ability of malignant tumor cells to invade surrounding normal tissues in vivo (7).

At about the same time, other investigators reported that several agents capable of producing tumors in vivo also produced changes in cells in vitro that caused them to resemble malignant cells (15, 16, 33, 43-46, 47, 52). After exposure to the transforming agent, the cells would begin to grow in a pattern in which they obviously crisscrossed one another or were in some way less orderly in their arrangement than untransformed cells. And whereas untransformed cells are typically monolayered, the transformed cells became multilayered. These alterations in culture pattern were generally explained as being the result of a failure of the cells to exhibit contact inhibition (see reference 43, especially p. 48; reference 44, p. 2).

One of the few reported attempts to make direct observations on transformed cell behavior was that of Vogt and Dulbecco (53). Those authors found that transformed cells, in contrast to normal cells, maintain their original direction of movement "when they touch and crawl over each other" (reference 53, p. 368). Although the observations were not detailed, that report at least represented an attempt to observe directly the social behavior of transformed cells.

The more common approach seems to have been to base judgments about cell behavior on observations of culture patterns at relatively low magnification, viewed once after a few days in culture. The presence of a pattern of crisscrossed multilayers has generally been assumed to indicate that the cells formed such a pattern by crawling over each other's surfaces. Therefore, the pattern alone was taken to indicate that the cells lacked contact inhibition of movement (see reference 14, p. 383).

In light of the importance of understanding the changes in cell behavior that take place in transformation to the malignant state, it seemed important to test these assumptions directly. This paper reports a series of observational and experimental studies carried out to determine how transformed and nontransformed cells differ in their behavior in culture and, more specifically, to determine to what extent the differences in culture pattern are the result of differences in the cells' capacity to exhibit contact inhibition of movement. Part of this work has already been reported in a preliminary note (12).

MATERIALS AND METHODS

Cell Culture

The 3T3 and Py3T3 cells used in this investigation were derived from a single culture of each type, kindly supplied by Dr. Howard Green (Massachusetts Institute of Technology). Upon receipt, the cells were subcultured and, at various times, aliquots of cells were frozen. All of the cells subsequently used were derived from early culture generations and were routinely cultured in Dulbecco's modified Eagle's medium (Gibco Diagnostics, the Mogul Corp., Chagrin Falls, Ohio), supplemented with 10% calf serum (Gibco) and antibiotics (50 U of penicillin and 50 mg of streptomycin/ml, hereafter abbreviated DECS). Stocks were maintained in plastic tissue culture flasks (Falcon 3024, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in a 5% CO_2 atmosphere at 37°C.

Time-Lapse Cinemicrography

Films were made with either a Bolex 16-mm camera with a Sage intervalometer or an Arriflex 16-mm camera and intervalometer, mounted on either a Zeiss Universal or a Nikon inverted microscope (model M). The intervals used for filming varied from 1 to 60 s, with an exposure of 0.5 s. The film used was Kodak Plus-X Reversal, Type 7276, processed commercially. All filming was done with phase contrast optics in a 37°C warm room. Films were projected for analysis with a Photo-optical Data Analyzer (L-W International, Woodland Hills, Calif.)

Culture Chambers Used for Filming

Two types of culture chambers were employed during time-lapse filming. For survey filming, cells were grown in 35-mm plastic Falcon tissue culture dishes (type 3001). These dishes were attached to the stage of an inverted microscope with double stick tape and enclosed in a small plastic housing into which 5% CO_2 was continuously fed, after bubbling through water.

For filming at higher magnifications or whenever better optical properties were desired, a thin filming slide was used. The slide consisted of a rectangular piece of aluminum or glass ($3 \times 1\frac{1}{2}$ in $\times$ <2 mm) with a hole in the center. A clean coverslip was fastened over one side of the hole with a ring of silicone stopcock grease (Corning Glass Works, Science Products Div., Corning, N.Y.), and a second ring of grease was placed around the hole on the opposite side of the slide. The assembled slide was placed in a Petri dish and autoclaved. Just before use, the hole was filled with culture medium, and a glass coverslip bearing the cells was inverted over it and pressed down. The exposed side of the culture-bearing coverslip was quickly rinsed with distilled water to wash off any adhering culture medium, because the salts and proteins would crystallize on drying and obscure the cells. The assembled chamber was then placed on the

microscope stage with the cells inverted. The chamber was occasionally used with an inverted microscope, in which case the chamber was placed on the microscope stage with the cells down. This type of chamber was found to have excellent optical properties. It is possible, for example, to oil both the objective and condenser lenses to the chamber, permitting maximum resolution. Glass slides (Bellco Glass, Inc., Vineland, N.J. or custom made) were found to work best, and cells survived in them for several days without requiring change of the medium. In aluminum slides, the cells looked unhealthy after 12–24 h.

Criteria Used to Determine the Relative Vertical Position of Cells: Overlapping Versus Underlapping

In this work it has been necessary to determine whether cells are moving under or over other cells. The most unambiguous way to determine the relative vertical position of cells is to use high magnification objective lenses with a shallow depth of field to make optical sections of the cultures (see Figs. 5 and 8). With low magnification lenses, all of the cells in subconfluent cultures lie within the same plane of focus; in such cases, other means are required to determine relative cell positions.

An alternative method is observing the behavior of the cell nucleus. The nuclear region of cells is thicker than the surrounding cytoplasm. As a result, the nucleus of a cell that is underlapping another cell often "hangs up" on the edge of the cell being underlapped. When the nucleus does underlap, it may become flattened or deformed in other ways as it squeezes under the other cell. The cell or process being underlapped may also become distorted and it may bow or bend around the underlapping nucleus. Aspects of such nuclear underlapping may be seen in Figs. 7 and 8.

Changes in the ruffling behavior of a cell may also indicate underlapping. Ruffling is due to the vertical uplifting of portions of the cell margin (26, 32). When a cell moves under another cell, ruffling usually stops, apparently because the margin is unable to lift up in the narrow space between the cell being underlapped and the substratum. The margin may continue to advance, however, and, as soon as the leading edge emerges from the other side, ruffles are seen again (Fig. 8).

Analysis of the Outcome of Cell Contact

Films of cells contacting each other were viewed, and one-to-one cell interactions were analyzed. Each interaction was scored as to the nature of the marginal areas that contacted and the outcome of the contact. Cell margins were classified as either locomotory or inactive. Locomotory margins were those showing protrusion-withdrawal activity at the time of contact. All other margins were classified as inactive. Outcomes were scored for continuation or cessation of locomotion after contact and for overlapping versus underlapping (see Table II).

Photomicrography

Still photomicrographs of cells were made with a Wild 35-mm camera and Kodak Plus-X film. Otherwise, the equipment and cultures were the same as those used for the time-lapse filming.

Electron Microscopy

Cells for electron microscopy were grown in 35-mm plastic tissue culture dishes (Falcon type 3001) and fixed *in situ* at 37°C for 15 min by replacing the medium with a fixative of 1% paraformaldehyde (Fisher Scientific Co., Pittsburgh, Pa.) and 2% glutaraldehyde (Polysciences Inc., Warrington, Pa.) in 0.1 M sodium cacodylate (Fisher Scientific) at pH 7.2, supplemented with 0.075% $CaCl_2$.

Cultures were postfixed in osmium tetroxide in 0.1 M cacodylate buffer, dehydrated to 90% ethanol, and left overnight at 4°C. Dehydration was completed at room temperature with four changes of 100% ethanol, to which aluminasilicate beads (Fisher "Molecular Sieves") had been added to remove any water. Ethanol was then replaced with a 1:1 mixture of 100% ethanol and Epon 812 working solution (23) for 60 min, followed by three changes of pure Epon 812 working solution for 30 min each. The final change of Epon was drained until a layer less than 1-mm thick remained. The dishes were left overnight in a dessicator under gentle vacuum (water aspirator) and the next day transferred to an oven at 45°C, for 24–48 h. Sections perpendicular to the substratum were made with the method of Eguchi and Okada (23).

Sections, picked up on uncoated copper grids of 300- or 400-mesh, were stained with Reynolds lead citrate and uranyl acetate, and viewed with either an RCA EMU-3 or a Hitachi 8S transmission electron microscope.

Identification of Cells in Mixed Cultures

Two methods were employed to distinguish one cell type from the other in mixed populations: labeling with carmine and seeding suspended cells of one type into an established culture of the other type.

CARMINE LABELING OF CELLS: A 0.1% (wt/vol) solution of Alum Lake Carmine (Fisher Scientific) dissolved in DECS medium was added to an established culture of cells at subconfluent density. After 24 h, the carmine medium was removed and the flask was rinsed once with fresh medium. The cells were then suspended, rinsed once with DECS, and plated into a fresh flask. The next day, the cells were again suspended, rinsed three times, and finally resuspended in medium and either added to the already spread cultures of cells of the other type or mixed with suspended unlabeled cells and plated together.

CELL SEEDING: A suspension of cells was added to a subconfluent established culture of the other type in a tissue culture dish or filming chamber. The chamber was then placed on the stage of an inverted microscope, a field was selected containing one or more spread cells, and filming was begun at once. In this way, the rounded cells of the added cell type could be observed as they settled on the substratum among the spread cells of the other type originally present and began to spread themselves. Thus, later in the film, the identity of any cell could be determined by retracing the sequence.

Determination of Marginal Adhesions

To determine the proportion of cell periphery that was adhering to the substratum, I used the following sampling method (also see Fig. 9). A polar grid, composed of 18 radii of a circle, intersecting at a common center and separated by even spaces of 20° of arc, was drawn on a piece of transparent plastic. This was placed over enlarged 35-mm photomicrographs of cells so that the grid was centered on the cell nucleus but was otherwise randomly oriented. The point where each of the 18 radii intersected the margin of the cell was scored as to whether or not it was adhering to the substratum. These determinations were based on the observations of Harris (28) which showed that smoothly curved, concave portions of the cell margin are free of substratum adhesion sites, whereas irregular or convex portions are areas where substratum adhesions are located. If the grid line intersected the margin at a site where the cell adhered to another, this site was counted as an adhering point. The number of intersected points that were scored as adhering, converted to a percentage of the total number of sampling lines, was taken as a measure of the percentage of the entire cell margin adhering to the substratum.

RESULTS

Crisscrossing Versus Monolayering in Py3T3 and 3T3 Cells—Description of the System

Fig. 1 shows the characteristic culture pattern of 3T3 cells. The pattern becomes apparent at low densities, as small groups of cells come together to form monolayered patches. The outlines of the individual 3T3 cells are obscured where the cells are in contact. The nuclei are evenly distributed and do not overlap one another, as expected for a cell monolayer. As the cell density increases, the pattern remains the same. In confluent cultures, viewed at low magnification, the cells appear to be in contact with each other all around their peripheries; but, because the cytoplasm of the cells is fairly transparent and produces a low contrast image, it is quite difficult to see the outlines of the individual cells. This gives one the impression that the cells in the monolayer are tightly apposed to one another like cobblestones in a pavement. However, careful observation reveals that the cells are actually superimposed on one another to varying degrees, particularly at their margins. Electron micrographs of vertical sections through confluent 3T3 cultures confirm that cytoplasmic structures exist in several thin layers (50 and Fig. 2).

This interweaving of cells explains the apparent decrease in cell size that occurs with increasing density (Fig. 1). Measurements of cell volume (13) show no decrease in volume with increasing density, nor is the apparent decrease in the area of

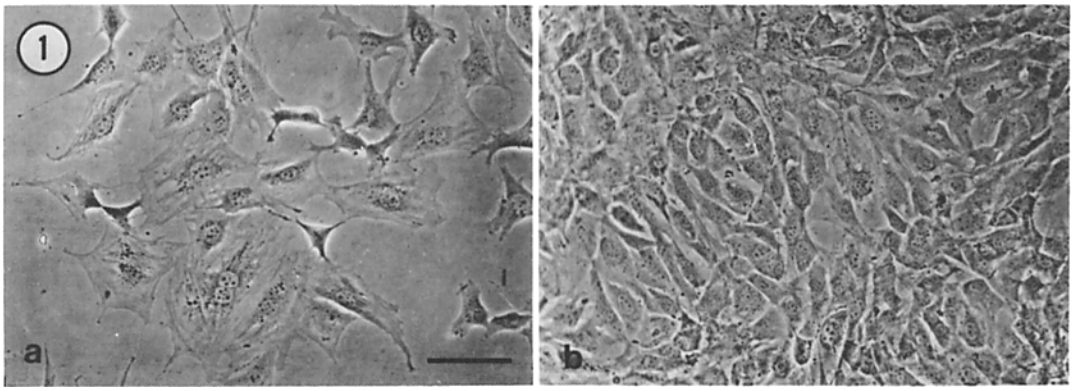

FIGURE 1 3T3 cells at low and high densities. Phase-contrast photomicrographs of two different living cultures of 3T3 cells. (*a*) Subconfluent density (100 cells/mm²). Several large, well-spread cells are present in this field, and the central group of cells has formed a small "monolayer." Some cell crisscrossing, however, is seen. (*b*) Dense culture (>550 cells/mm²). Cells are crowded together. There are few nuclear overlaps but cell crisscrossing may be seen in places where the cells are relatively more transparent. Each cell appears to occupy less area than in *a*. This may be the case, or else the cells may be as spread as those at low density but surrounding cells are extensively superimposed. Bar, 100 μm. × 100.

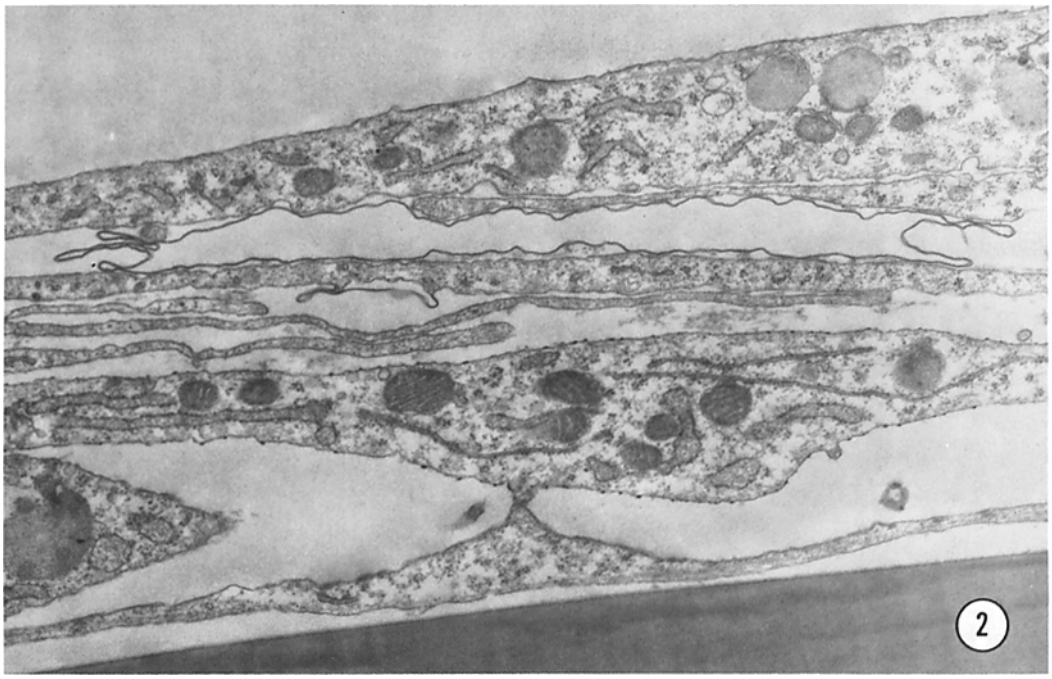

FIGURE 2 Vertical section through confluent 3T3 culture. Electron micrograph showing the interleaving of 3T3 cytoplasmic processes. In this section, thicker, organelle-containing processes crisscross with thinner processes, containing only filamentous structures and ribosomes. × 15,700.

3T3 cells in confluent cultures due to an increase in cell thickness, because thin-section electron micrographs reveal no marked increases in cell thickness (Fig. 2).

The superposition of the cytoplasmic structures of 3T3 cells does not extend to the distribution of cell nuclei. Nuclear overlap ratios from fixed cultures of 3T3 cells (Table I) indicate a highly nonrandom distribution of cell nuclei; nuclei are distributed in a strict monolayer. Nuclear overlap determinations can, therefore, be misleading—on the basis of such measurements, 3T3 cultures give the overall impression that they are monolayers when, in fact, they are not.

In contrast to their nontransformed counterparts, Py3T3 cells exhibit a pattern of extensive crisscrossing. As shown in Fig. 3, this pattern becomes apparent at low cell densities and is the result of both the interweaving of numerous thin cell processes from individual multipolar cells and the superposition of cell bodies. The pattern is most clearly seen in cultures that consist of a continuous network of contacting cells. At high cell density, the pattern of crisscrossing becomes obscured, as the cells become crowded into dense multilayered arrays.

TABLE I

Nuclear Overlap Ratio (O/E)

3T3	Py3T3
0.096	0.4222

Nuclear overlap ratios were determined by the method of Weston and Hendricks (56).

O = observed overlaps; E = expected overlaps where $E = [2a(n - 1)/b]$ and where a is the number of hits on nuclei by the random dots of a Chalkley grid, n is the number of cells, and b is the total number of grid dots.

Whereas the cytoplasm of 3T3 cells is rather transparent, that of Py3T3 cells shows up distinctly in phase contrast. Py3T3 cell processes are also outlined by bright phase-contrast halos. As a result, the cell outlines are readily observed and cell overlaps show up clearly. This pattern of overlapping is confirmed by electron micrographs of vertical sections through Py3T3 cultures (13, 50). In addition, the pattern of crisscrossing of Py3T3 cells is associated with a greater amount of nuclear overlapping than is seen in 3T3 cells but the amount of nuclear overlapping is still less than would be expected if nuclear distribution were random (Table I).

3T3 Cell Movement, Contact, and Monolayer Formation

3T3 cells in culture are well spread and as they move about on the substratum they contact one another extensively over broad regions of their margins. This contact between the cells results in interference with the direction of cell motility, such that the bottom of the culture dish is eventually covered with a layer of highly spread and fairly evenly distributed cells. An example of this behavior is shown in Fig. 4, in which the direction of cell movement changes after contact between moving cells. (Note that "contact" is used in the strictly operational sense of no space being visible between the cells at the level of resolution used. It does not imply adhesion.) Contact between 3T3 cells is often accompanied by a cessation of visible ruffling activity and the formation of adhesions between the cells. It is important to note that intercellular adhesions are only convincingly demonstrated by the drawing out of retraction fibers between two cells as they separate after contact, and this criterion is used throughout this study.

The effects of cell contact are highly localized in that locomotory activity and ruffling cease only in the region of contact (e.g., Fig. 4). Locomotory activity, mostly with associated ruffling, may continue along noncontacting portions of the cell margin, eventually resulting in the migration of contacting cells away from each other.

An important question to ask about the contact interactions between 3T3 cells is whether or not the cells migrate *over* one another. The answer is unqualified. In the course of this investigation, no 3T3 cell has ever been observed to use another as a substratum for locomotion. This statement is based on careful observation of dozens of filmed sequences involving literally hundreds of individual contact events, and it confirms the conclusion of other investigators that cells that monolayer do not overlap (6).

But, as shown above, 3T3 cell populations are at least partially multilayered. How, then, in the absence of overlapping does the superposition of cell bodies occur? Examination of time-lapse films reveals numerous instances of 3T3 cells underlapping one another. Indeed, in every instance where one cell becomes superimposed on another, careful observation has revealed it to be the result of underlapping (Fig. 5).

The contribution of these various types of cell behavior to the production of the overall culture pattern of 3T3 cells can be seen in Fig. 6. A patch of "monolayer" forms as a result of the cells mutually interfering with each other's direction of locomotion. Overlapping does not occur, and underlapping, though common, is limited by the degree to which the cells inhibit each other's locomotion. Inasmuch as cells in the center of the patch continue to translocate, it is clear that monolayering is due to restriction on the *direction* of cell

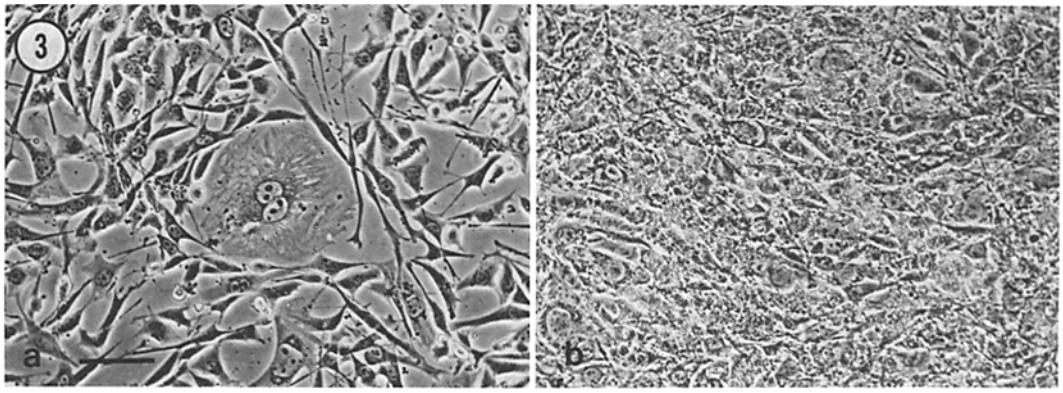

FIGURE 3 Py3T3 cells at low and high densities. Phase-contrast photomicrographs of two different living cultures. (*a*) Subconfluent density (575 cells/mm^2). Most cells are in contact with several other cells, but, owing to their spindly shapes, extensive open areas are still present in the culture. 3T3 cells at the same density almost completely cover the available substratum (Fig. 2). This is a typical crisscrossing pattern. A large binucleated cell is present in the center of the field. Note the failure of the surrounding cells to use the surface of this cell as a substratum. (*b*) Dense culture. The cells are in a multilayer, and the cell pattern is obscured by the large number of cells present. Bar, 100 μm. × 100.

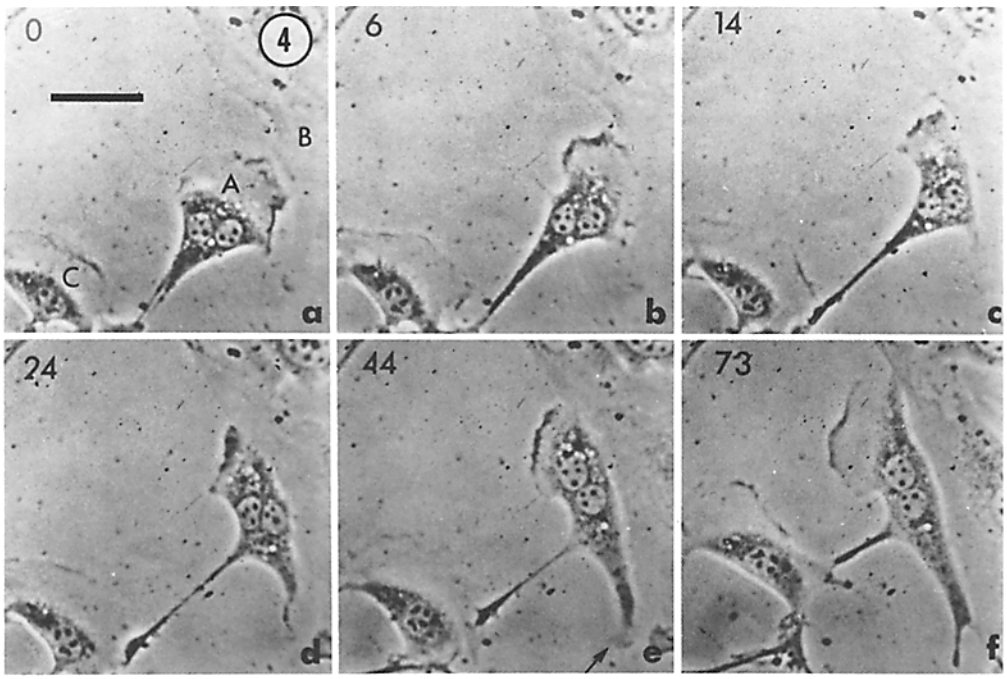

FIGURE 4 3T3 cells moving and contacting each other with subsequent changes in the direction of movement. Single frames from a time-lapse film. Time given in minutes elapsed after first frame. Bar, 50 μm. × 250. (*a*) 0 – Cell *A* (binucleate) is migrating toward the upper right behind a ruffling lamellipodium, drawing out retraction fibers behind it. (*b*) 6 – Cell *A* has contacted cell *B* along a broad front (~60 μm). Cell *A* ceased ruffling along the region of contact, but continues to ruffle elsewhere. (*c*) 14 – Cell *A* has broadened its area of contact with cell *B*, as it continues to extend its margin toward 11 o'clock. Now cell *C* has contacted the "tail" of cell *A*. (*d*) 24 – Cell *A* is continuing to spread along the margin of cell *B*. Its long axis has shifted as the margin has advanced toward both 11 and 5 o'clock. The locomotory activity of cell *C* has apparently stopped where it is contacting cell *A*. (*e*) 44 – The main ruffling lamellipodium of cell *A* has begun to extend away from cell *B*. A second, smaller lamellipodium has expanded at the opposite end of cell *A* (arrow). The tail of *A* is adhering to cell *C* and has shortened as cell *C* moves toward cell *A*. (*f*) 73 – The main lamellipodium of cell *A* has broadened while the small lamellipodium has become dormant and cell *A* has begun to pull away from cell *B*. Meanwhile, cell *C* has continued to advance upfield, pulling the "tail" of cell *A* along with it. Note that cell *C* has changed direction as a result of contacting cell *A*.

movement and not on the ability to move. Cell *B* in Fig. 6, for example, continues to migrate even though at times it is surrounded by other cells. Thus, although cell locomotion continues in situations where cells are in contact, the cells fail to utilize the surfaces of their neighbors as substrata for movement. All of their movements are confined to the inanimate substratum of the culture chamber where they are limited and directed by lateral contact interactions with other cells.

Py3T3 Cell Movement, Contact, and Crisscrossing

Films of Py3T3 cultures reveal that these cells translocate over the substratum largely as a result of the extension of variable numbers of individual cell processes, each of which apparently behaves independently. As these cells and their processes extend over the substratum, they produce the patterns of crisscrossing characteristic of populations of Py3T3 cells. However, at no time has a Py3T3 cell or one of its processes been observed to migrate *over* the surface of another cell. Rather, in every instance where it has been possible to make a clear judgment (totaling dozens of contact events), crisscrossing is found to result exclusively from the cells extending *under* other cells, i.e., from *underlapping*.

The results of Py3T3 underlapping behavior are shown in Fig. 7. This figure shows how it is possi-

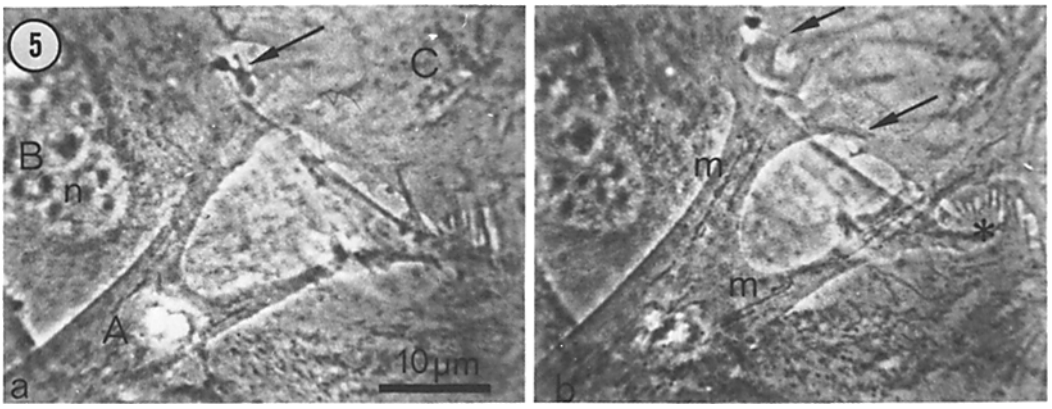

FIGURE 5 3T3 underlapping. Single frames from a time-lapse film. (*a*) 3T3 cell (*A*) underlapping another 3T3 cell (*B*). The relative vertical positions of the cells were determined unambiguously by focusing. Note that the outline of *A* is in focus while the nucleus (*n*) of the overlying cell *B* is out of focus. Note also the ruffle from cell *A* (arrow) extending upward through a gap between cells *B* and *C*. (*b*) The same field 25 min later. Cell *A* has advanced toward 1 o'clock and is now underlapping cell *C* as well (arrows). Mitochondria (*m*) may be seen in focus in cell *A*. Note the closely spaced adhesion points between cells *B* and *C* (asterisk). Bar, 10 μm. × 950.

ble to produce both crisscrossing patterns and nuclear overlaps solely by underlapping. Indeed, time-lapse films indicate that underlapping is one of the major modes of Py3T3 social behavior, contact between these cells often being followed by one cell moving under the other.

On some occasions, however, contact between Py3T3 cells is followed by a cessation of both ruffling and locomotory activity and the formation of adhesions (Fig. 8). This result of cell contact is identical with that seen between 3T3 cells and between other types of cells showing contact inhibition of locomotion (4, 21, 51), and it is most often observed when two Py3T3 cells contact each other via their lamellipodia.

As shown in Fig. 8, the direction of movement of individual Py3T3 cells is channeled by their contact interactions with other cells. Movement in the direction that brought about the contact persists only when the cell *underlaps* the cell that it is contacting; otherwise, further movement in that direction is prevented. This directional inhibition of locomotion is usually associated with the cessation of both ruffling and locomotory activity and the formation of adhesions. Another phenomenon often seen accompanying cell contact is "contact retraction," so called because the two contacting cells are seen to pull away or retract from each other (1, 2, 55; see also Fig. 8: 26 and 40 min). Contact retraction apparently occurs only when two cells adhere to each other. Therefore, the failure of underlapping cells to show contact retraction is consistent with the conclusion that underlapping cells make no adhesions with the cells overlying them.

Thus, in terms of their contact behavior, Py3T3 cells are remarkably like 3T3 cells. Both 3T3 and Py3T3 cells fail to move over each other's surfaces; and, in both cell types, contact may result in the cessation of locomotory and ruffling activities and lead to the formation of cell adhesions. Both cell types also exhibit underlapping behavior. However, Py3T3 cells appear to underlap to a much greater extent than 3T3 cells, with the resulting formation of crisscrossed patterns. Thus, it seems reasonable to conclude that the differences in their culture patterns are not the result of any qualitative differences in their contact behavior. In particular, it now seems clear that the assumption that Py3T3 cells crawl over one another whereas 3T3 cells do not is erroneous. Rather, it appears likely that the behavioral differences between these two cell types are quantitative.

Quantitative Comparison of Cell Contact Behavior

To determine whether there are quantitative differences in the contact behavior of 3T3 and Py3T3 cells, I analyzed every cell contact event that could be followed to completion in films and recorded the outcomes. As shown in part A of Table II, there are significant differences between

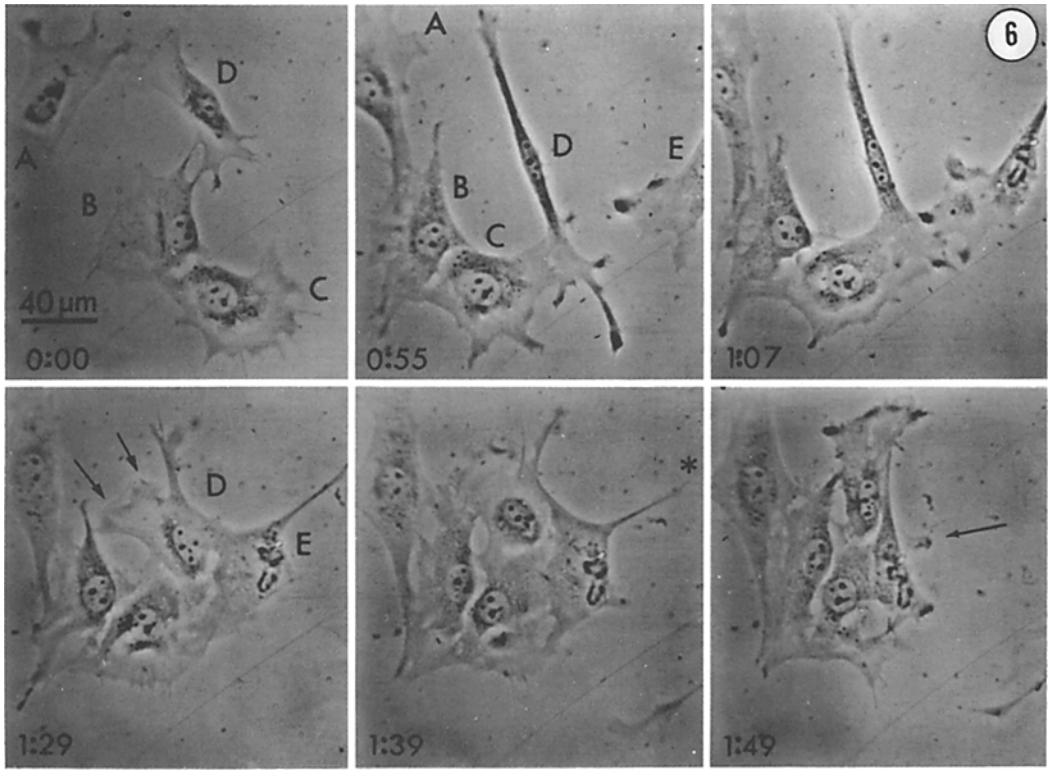

FIGURE 6 "Monolayer" formation by 3T3 cells. Single frames from a time-lapse film. Time is given in hours and minutes elapsed from the first frame. Bar, 40 μm. × 250. (0:00) An area of 3T3 culture with four individual cells is shown. The long scratch in the plastic substratum curving across the field between cells *B* and *C* serves as a reference mark in subsequent frames. (0:55) Cells *B* and *C* have moved to the left maintaining and broadening their contact area while cell *A* has extended a lamellipodium down and contacted cell *B*. Cell *D* has become highly elongated as a result of the movement of lamellipodia at opposite sides of the cell. This is an unusual configuration for a 3T3 cell but illustrates the ability of cells to undergo marked changes in shape over a period of time. Cell *E* is entering the field from the right. (1:07) All five cells are now in continuous contact. (1:29) Cells *A*, *B*, and *C* have remained relatively stationary with slight changes in shape as a result of marginal locomotor activity. The end of cell *D* is retracting from 12 o'clock, and its advance toward 5 o'clock has been blocked by contact with cell *E*. Meanwhile, a new lamellipodium (arrows) has formed from the stretched side of the cell, causing cell *D* to become more polygonal in shape. Cell *E* has continued to advance toward 9 o'clock, spreading out along the sides of cells *C* and *D*. (1:39) The cells have moved closer together, expanding their contact areas and forming a small patch of "monolayer." Note the retraction fiber that cell *E* has pulled out behind itself (asterisk). Also note that the lamellipodium of cell *D* has shifted its direction of movement from 10 to 11 o'clock as a result of contact with cell *B*. (1:49) The retraction fiber of cell *E* has broken, causing the cell body to shift toward 9 o'clock and revealing the presence of a process of cell *C under* cell *E*. The cells of the apparent "monolayer" are thus shown to be superimposed to a certain extent. Also, the cells have continued to shift their positions within the group. Especially as cell *D* has moved further toward 12 o'clock, cells *B* and *C* have moved into the space vacated. Throughout, this movement has continued in spite of extensive areas of cell contact.

the two cell types ($\chi^2 = 11.32$, 1 df, $P < 0.05$). Although neither cell type overlaps at all, Py3T3 cells underlap one another about twice as often as do 3T3 cells. Conversely, 3T3 cells stop moving on contact about twice as often as do Py3T3 cells.

The source of this difference becomes apparent if the contact events are divided into two types: (*a*) those occurring between the locomotory margins (lm) of two cells (lm-to-lm) and (*b*) those occurring between the locomotory margin of one

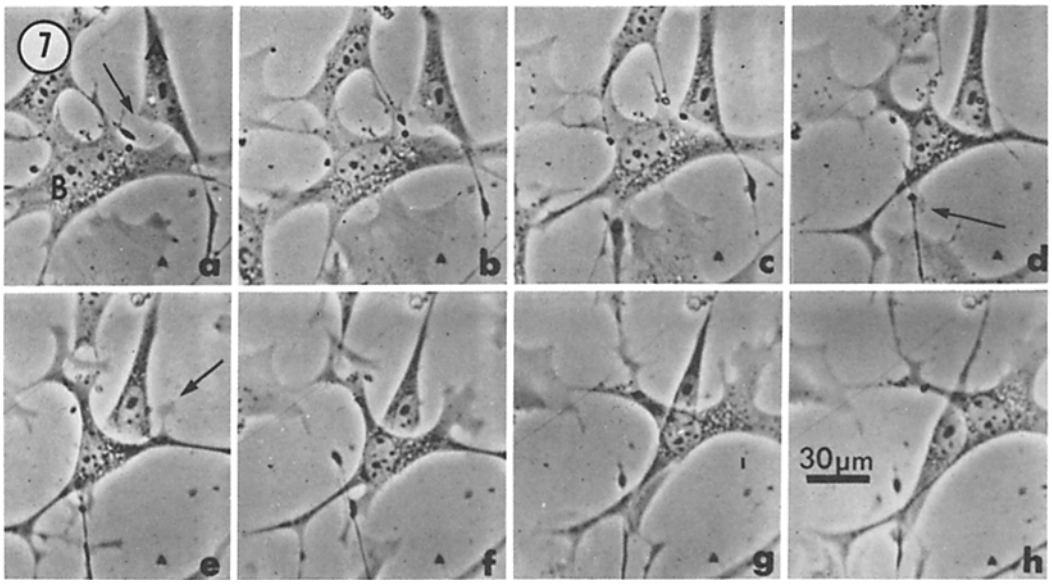

FIGURE 7 Py3T3 cells contacting and underlapping. Single frames from time-lapse film. Elapsed time given in hours and minutes. Bar, 30 μm. × 260. (*a*) 0:00—Cell *A* has begun to extend a process (arrow) toward cell *B*. (*b*) 0:37—*A* has continued to extend toward *B*. (*c*) 1:19—*A* and *B* are in visible contact. The cell body of *A* has moved closer to *B*. (*d*) 2:22—The process of *A* has emerged from under *B* (arrow) on the far side. (*e*) 2:46—The process of *A* has extended farther over the substratum and the body of *A* has followed in the same direction. Cell *B* has become quite concave and pulled back in the region where *A* is underlapping, but meanwhile a new protrusion of *B* (arrow) has formed from the side of an extended process to the right. (*f*) 3:30—The nucleus of *A* has extended slightly under *B*. Meanwhile the body of *B* has begun to shift toward 2 o'clock behind the expanding new process. (*g*) 4:24—The combination of *A* moving toward 7 o'clock under *B* with the concomitant shifting of *B* toward 2 o'clock behind its new extending process has produced a nuclear overlap. (*h*) 4:43—*B* has shifted further toward 2 o'clock, leaving *A* extending under it.

cell and the inactive margin (im) of another (lm-to-im). See Materials and Methods for definition.

Considering first the lm-to-lm contacts (Table II B), it can be seen that 75% of such contacts between 3T3 cells result in both cells stopping their movement, whereas 25% result in one stopping and one underlapping. For Py3T3 cells, the respective figures are 67 and 33%. These figures are not statistically different for the two cell types ($\chi^2 = 0.089$, 1 df, $P \simeq 0.75$). Thus, for both 3T3 and Py3T3 cells, the most frequent outcome of contact between two locomotory margins (i.e., between two lamellipodia) is mutual cessation of locomotion. Now, consider what happens if the contact is lm-to-im (Table II C). 3T3 cells stop in 54% of the cases and underlap in 46%. Py3T3 cells, in contrast, stop only 14% of the time and underlap 86% of the time. Therefore, in contacts between a locomotory margin of one cell and an inactive margin of another, Py3T3 cells overlap significantly more often than do 3T3 cells ($\chi^2 = 14.25$, 1 df, $P < 0.005$). Moreover, in all of the contact events analyzed, lm-to-im contacts occurred more frequently than lm-to-lm in both cell types. And although for 3T3 cells this does not constitute a departure from randomness (24 vs. 20), Py3T3 cells show a significant tendency for lm-to-im contacts to occur more frequently (73 vs. 40; $\chi^2 = 9.06$, 1 df, $P < 0.005$). These results indicate, therefore, that Py3T3 cells underlap more often than 3T3 cells because (*a*) Py3T3 cells have a greater frequency of lm-to-im contacts, and (*b*) a greater proportion of such contacts result in underlapping in Py3T3 cells than in 3T3.

Distribution of Marginal Areas Adhering to the Substratum

The quantitative data just presented indicate an increased tendency of Py3T3 cells to underlap one

another. Why this should be so is suggested by observations from time-lapse films that Py3T3 cells underlap the stretched, nonadhering margins of other cells. Margins that lack adhesions are also inactive margins.

For a cell to assume a typical spread morphology, it must adhere to a suitable solid support and the adhesions between a cell and the substratum appear to be located largely at or near the cell margin (28, 37). It is the pattern of these marginal adhesion sites that is largely responsible for the shape of the cell. In fact, a cell may be thought of as being stretched among its various adhesion sites; for, if one site is broken, either naturally or by micromanipulation, the cell snaps back to a new shape based on the pattern of the remaining adhesion sites (28). Because adhesions between a cell and the substratum could very well block other cells from underlapping, underlapping would be favored along regions of the cell margin where substratum adhesions are absent.

I therefore decided to examine the distribution of cell-substratum adhesions. To do this, I used a sampling method in which 18 points on the cell margin were selected with an overlying polar grid (see Materials and Methods), and each point was scored as being free or not free of substratum adhesions (see Fig. 9). This method does not necessarily detect individual sites of adhesion but, rather, areas of the cell margin where adhesion sites are presumed to occur because the margin has a convex or irregular contour. Marginal areas that are concave in contour are scored as being nonadhering (28). Measurements were made on photomicrographs of 3T3 and Py3T3 cells selected at random. The results (Fig. 10) indicate that, on the average, 3T3 cells show twice as much margin adhering to the substratum as Py3T3 cells—50 as compared to 22%.

In addition to having a greater percentage of their margins adhering to the substratum, 3T3 cells have adhesions that are distributed in a more continuous way. A measure of this is the number of adjacent lines of the sampling grid (Fig. 9) which intersect adhering areas of the cell margin. Table III shows the frequency (as a percentage of the total number of adhesion points detected) with which sampled adhering points occur in various-sized groups of consecutive "hits" on adhering areas. 48% of the Py3T3 adhesion sites sampled occurred singly, i.e., without adjacent sampled points also adhering, and 30% occurred in pairs. On the other hand, for 3T3 cells only 9% of the adhesion sites occurred singly; 19% were in pairs, 16% in groups of three, and 12% in groups of four. In one 3T3 cell, 16 adjacent sampled points of the margin were in adhering areas.

Contact between 3T3 and Py3T3 Cells

All of the preceding observations on cell contact pertain to interactions between cells of the same type, but contact interactions between 3T3 and Py3T3 cells are perhaps of even more significance because of their possible relevance to the problem of invasiveness. For this reason, contact interactions between 3T3 and Py3T3 cells were observed in mixed cultures. Inasmuch as some cells of each type are always similar in morphology to cells of the other type, one of two methods was used to distinguish the cells: carmine labeling or addition of a suspension of one type of cells to a spread culture of the other type (see Materials and Methods). The outcome of heterotypic contacts between 3T3 and Py3T3 cells is essentially the same as that of contacts between cells of the same type. After contact, cells either underlap or cease moving. Py3T3 cells readily underlap the larger, spread 3T3 cells. The reverse was observed less frequently, because the lamellipodia of 3T3 cells are usually too wide to avoid contacting adhering portions of Py3T3 cell margins. Mutual contact between locomotory areas of the two cell types results in a cessation of locomotory activity and ruffling and the formation of adhesions between the cells. No case was observed of a cell of one type moving over the surface of a cell of the other type. No quantitative measurements were made, but, as expected, Py3T3 cells appeared to underlap large 3T3 cells more frequently than to be inhibited by contact.

DISCUSSION

Contact Interaction and Cell Culture Pattern

The major finding of this investigation is that Py3T3 cells exhibit basically the same repertory of contact interactions as their nontransformed parent cells, 3T3. Contact between cells of both types results either (*a*) in the cessation of locomotion in the direction that produced the contact, or (*b*) in the continued movement of one cell *under* another. Neither 3T3 nor Py3T3 cells move over the upper surface of other cells. This finding is interesting in light of the assumption made by many

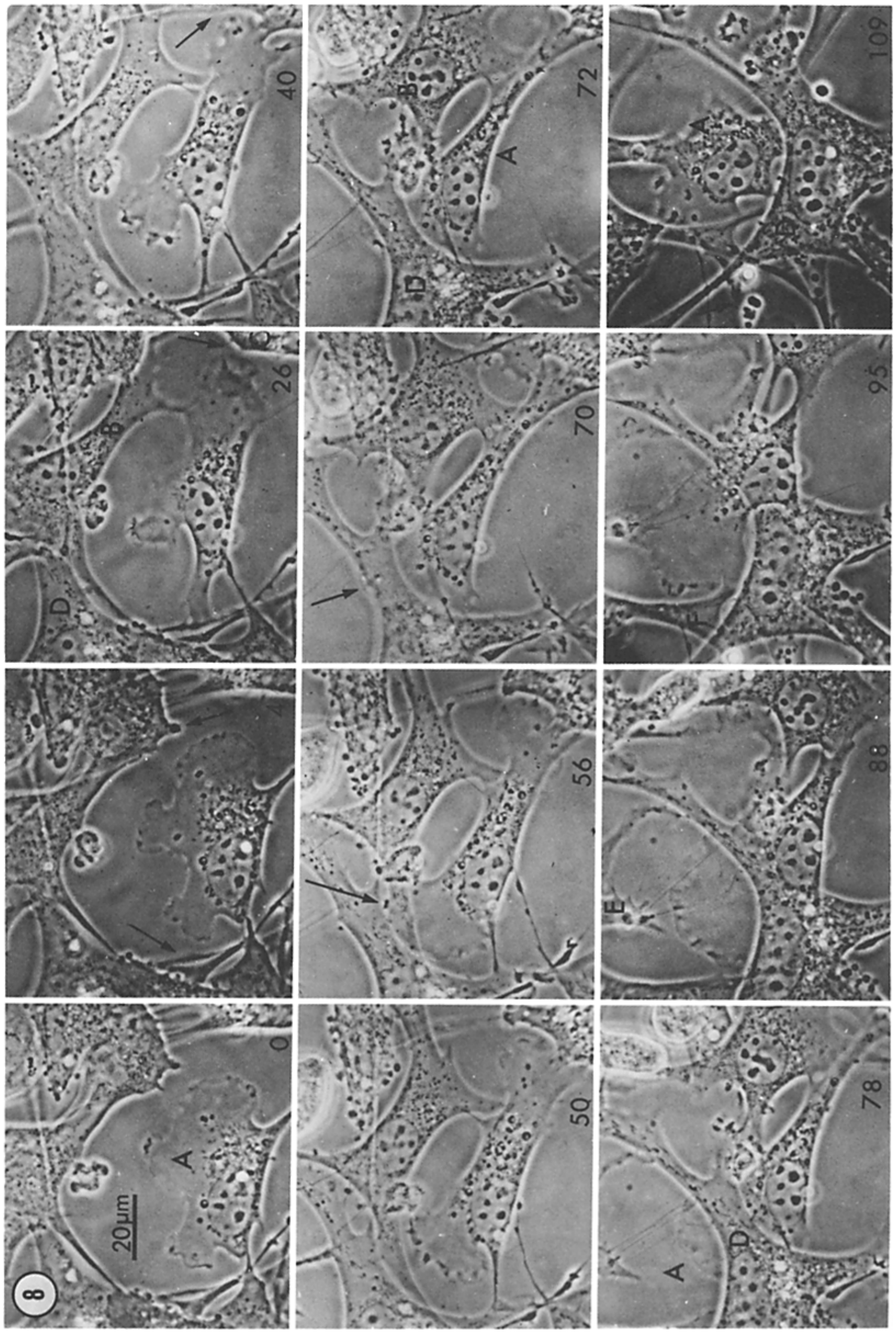
8
20μm
A
0
50
78
56
88
26
70
95
40
72
109
B
D
E
F

TABLE II
The Outcome of Contact between Cells

	3T3		Py3T3	
	Actual number	As % of total contact events	Actual number	As % of total contact events
A Total number of contact events analyzed	44		113	
Number resulting in one cell overlapping another	0		0	
Number resulting in one cell underlapping another	16	36	76	67
Number resulting in cessation of all locomotion	28	63	37	33
B Total number of cell contacts between two locomotory margins (lm)	20		40	
Locomotion of both cells stops	15	75	27	67
Locomotion of only one stops; the other underlaps	5	25	13	33
C Total number of contacts between the locomotory margin of one cell and the inactive margin of second cell (lm to im)	24		73	
Locomotion of first cell stops	13	54	10	14
Locomotion of first cell continues—*underlaps* second cell	11	46	63	86

The data in this table are based on scoring of contact events between pairs of 3T3 or Py3T3 cells (see Materials and Methods).

In part A, the first column under each cell type gives the number of events observed; the second column gives the percentage of total contact events which that number represents. In parts B and C, the first column gives the number of events observed, and the second gives the number as a percentage of the contact events falling within the B or C category.

FIGURE 8 Py3T3 cells moving and contacting each other. Single frames from a time-lapse film. Elapsed time given in minutes. Bar, 20 μm. × 500. (0) Cell *A* is migrating toward a group of other cells behind three ruffling areas of its margin. (4) Cell *A* has advanced upfield and is about to contact a cell process on the left (arrow) and an adhering margin of cell *B* to the right (arrow). (26) Both contacts have resulted in the formation of adhesions, and a cessation of marginal activity of cell *A* at the points of contact. Cells *A* and *B* have mutually retracted where they have contacted and adhered, as shown by their taut contours, indicating that they have been put under tension (contact retraction). Another marginal protrusion of cell *A* is about to contact cell *C* to the far right (arrow). (40) Mutual retraction at the area of contact is now more evident. Cell *A* continues to move upfield behind its single remaining major ruffling edge. A second active edge advances toward the side of cell *C* (arrow). (50) Cell *A* is about to contact the stretched side of *B* and at the left is pulling away from the contact made at 4–26 min above. Note the long retraction fiber. (56) Cell *A* has underlapped the extended process of cell *B* and a small flicker of a ruffle can be seen in the gap between cells *B* and *D* (arrow). (70) Cell *A* has continued to migrate under the extended processes of cells *B* and *D* and the tip of the protruding margin of *A* is seen emerging from under the far side of cell *D* (arrow). (72) Cell *A*'s margin has extended farther beyond cell *D* and is underlapping retraction fibers extending from cell *E* to cell *D*. The process of cell *B* has become more attenuated. (78) Cell *A* has continued to move from under *B* and *D*. Although the edge of *A* has continued to extend into the free area beyond, the thicker nuclear region of the cell has lagged behind, apparently unable to pass under the bridging process of cell *B*. Note that not only is the nucleus held up, but also the granular cytoplasmic material (organelles) associated with the nucleus. (88) The nuclear region of cell *A* has passed under *B* and is beginning to pass under cell *D*. Progress is apparently difficult, as the advancing margin of *A* has extended well beyond the nuclear region and is about to contact cell *E*. Note that two or more of the retraction fibers of cell *E* appear to be adhering to the top of cell *A*. Note also the total absence of perinuclear granular material in the spreading lamellar cytoplasm. (95) The nucleus of *A* is deformed as it squeezes under cell *D*. The margin of *A* has contacted cells *E* and *F* and has adhered to cell *F*. Note the first appearance of perinuclear granular material as it "squeezes" from under cell *D*. (109) The nucleus of *A* has emerged from under cell *D* and has quickly caught up with the advancing margin. Part of the margin has adhered to cell *E*, but another part has split off as a new process, underlapped processes of cells *F* and *D*, and is about to contact the ruffling margin of a third cell (arrow). Note that as the nucleus has caught up to the advancing edge, so has the granular cytoplasm.

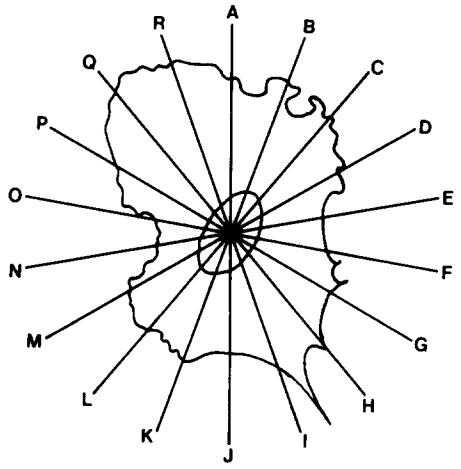

FIGURE 9 Margin sampling method. This tracing illustrates the method used to determine the proportion of the margin adhering to the substratum (see Materials and Methods). Here a cell has been drawn in, but in the actual assay, photomicrographs of cells were used. For the cell shown, lines *A–D, H,* and *K–R* intersect the margin at points where it is adhering to the substratum, whereas lines *E, F, G, I,* and *J* intersect adhesion-free areas of margin. Thus, for this cell, 13 out of the 18 points sampled are adhering to the margin, or 72%.

workers that a pattern of irregular crisscrossing of cells in culture is produced by the cells' crawling over each other's surfaces and is thus evidence of a deficiency in contact inhibition of movement (14–16, 43–45, 53). In the case of Py3T3 cells, this assumption is clearly false, and it is likely to be false for other cell types as well, because the assumption fails to take into consideration that crisscrossing can be produced either by underlapping or by overlapping. In Py3T3 cells, cell culture crisscrossing is due exclusively to underlapping.

Indeed, time-lapse observations of other cells indicate that underlapping is the way in which cells of many types cross each other in culture on a plane substratum. In films of the following cell types, I have seen many instances of underlapping without ever having seen a single instance of one cell crawling over the surface of another: sarcoma 180 cells (established line), chick heart fibroblasts, and murine sarcoma virus-transformed BALB/3T3 cells. The last cell type forms malignant tumors on transplantation to BALB mice (48). Other workers have observed underlapping in chick heart fibroblasts (17, 27, 57); in normal mouse embryo fibroblasts, murine sarcoma-transformed mouse fibroblasts, and neoplastic mouse fibroblasts (25); and in BHK21 and PyBHK21 cells (24). All of these observations were made with either time-lapse cinemicrography or scanning electron microscopy, two methods ideally suited for determining the relative positions of interacting cells. In light of these studies, the interactions of other transformed cells and tumor cells should be examined by these same methods to determine to what extent overlapping and underlapping behavior occurs. Only when several different types of cells have been studied in this manner will a basic understanding of the behavior of transformed cells in vitro be possible. But, in any event, it is clear that an end result, such as crisscrossing, cannot be taken as evidence of the mechanism that produced it.

One question that should be considered before going on is why the conclusions I reached differ so from those reached by others who have examined transformed cells. I believe the answer, at least in part, is that technical problems related to the viewing or filming of cells at low magnifications make it very difficult to tell whether or not differences in cell patterns are owing to qualitative differences in cell contact behavior. For example, the cytoplasmic multilayering that occurs in 3T3 cultures is very difficult to detect in the light microscope unless oil immersion lenses are used. This is probably because the highly spread 3T3 cells are quite thin and produce low contrast images. Therefore, it is hard to see clearly the outlines of cells that are under other cells. High magnification oil immersion optics are especially helpful when trying to distinguish underlapping from overlapping because the shallow depth of field afforded by such lenses enables one to make optical sections of the cells and thus determine unambiguously whether they are moving over or under each other. Good optics can also help one avoid another pitfall, which derives from using the behavior of ruffles as an indicator of a cell's locomotory activity. Although it is true that ruffling is usually associated with locomotory activity, it is also true that locomotory activity may occur with a total absence of ruffling (e.g., see reference 3, p. 348). When either 3T3 or Py3T3 cells underlap, ruffling stops, probably because the cell margin is mechanically prevented from lifting up by the cell "overhead." On the other hand, the lamellipodium of the underlapping cell continues to move forward, but this can only be seen when high resolution oil immersion optics are used. Thus, it seems to me

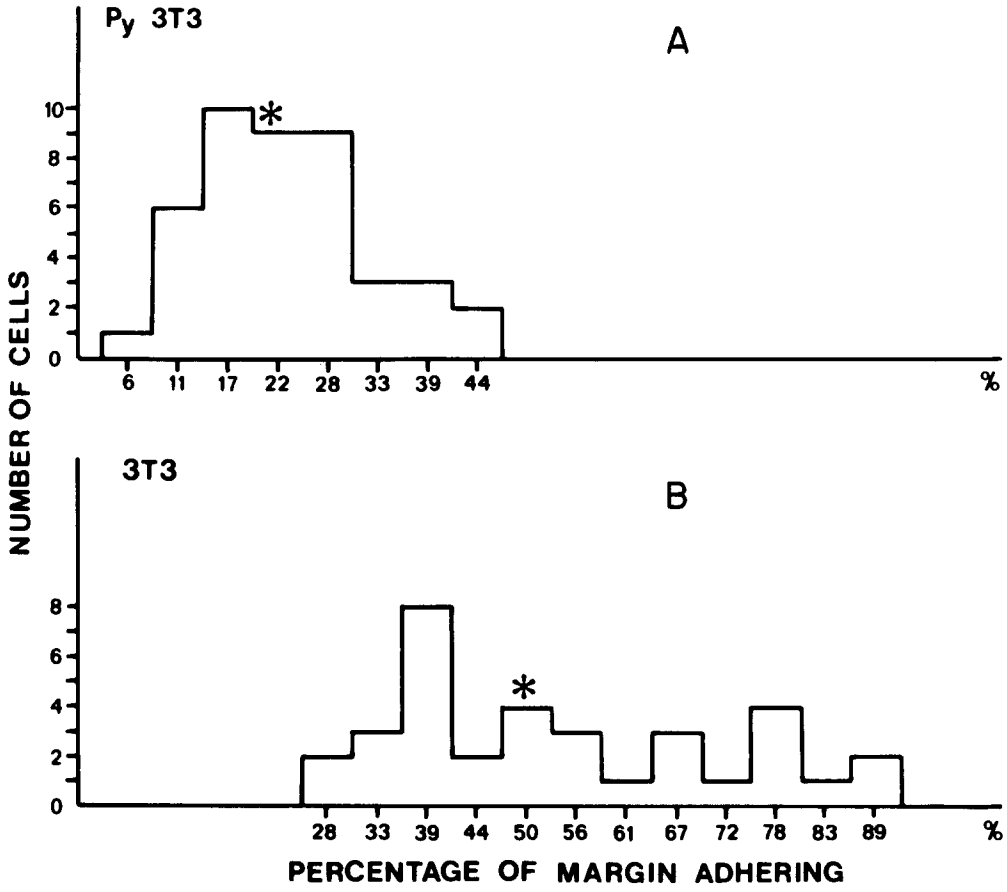

FIGURE 10 Marginal adhesions. These two histograms show the distribution of values for the percentage of the cell margin adhering to the substratum, using the sampling method illustrated in Fig. 9. The abscissa gives the percentage of the 18 lines of the sampling grid intersecting adhering points of the cell margin. The ordinate gives the number of cells. The median value is indicated by an asterisk. (A) Py3T3; median value = 22%. (B) 3T3; median value = 50%.

that the lesson to be drawn is that conclusions about cell behavior should be based only on direct observations with the best optics available.

Occurrence of Contact Inhibition in 3T3 and Py3T3 Cultures

Both 3T3 and Py3T3 cells show contact inhibition after some contacts, but after other contacts they do not. Are these observations in contradiction? The answer depends upon how one defines contact inhibition. If contact inhibition is defined as failure to overlap, then both 3T3 and Py3T3 cells show total contact inhibition. But this definition ignores the whole question of underlapping, which is a major aspect of the behavior of these cells. A more useful definition is the one proposed by Abercrombie and Heaysman (6) in their original study. These authors defined contact inhibition as a directional restriction of cell movement due to contact such that a cell fails to continue moving in the original direction that produced the contact. Contact refers here to what appears to be contact at the level of resolution of the light microscope. This definition provides an operationally useful description of the phenomenon and it also avoids confusing the *phenomenon* itself with the various *mechanisms* that might produce it. According to this definition, both 3T3 and Py3T3 cells can be said to exhibit contact inhibition because contact between cells of both types may result in directional restrictions on their movement. This directional restriction derives from the complete ab-

TABLE III

Distribution of Substratum Adhesion Sites

Group sizes of consecutive marginal adhesion sites	Percentage of total number of adhesion sites sampled falling in each sized group	
	3T3	Py3T3
	%	%
1	9	48
2	19	30
3	16	10
4	12	9
5	3	3
6	4	–
7	7	–
8	10	–
9	0	–
10	6	–
11	4	–
12	4	–
16	5	–

sence of overlapping and from a variable amount of underlapping. Py3T3 cells differ from 3T3 cells because in a greater proportion of their contacts Py3T3 cells underlap, thus effectively circumventing directional restriction on their movement. Thus, Py3T3 cells exhibit a lower frequency of contact inhibition.

These results confirm the importance of contact inhibition in controlling cell behavior in culture, but they challenge the hypothesis that transformed and tumor cells necessarily differ from normal cells in their *ability* to exhibit contact inhibition. In most of those contacts in which locomotion is not inhibited, both 3T3 and Py3T3 cells avoid being contact-inhibited by underlapping the portions of the cell margin that are unattached to the substratum. It seems possible that during such underlapping the two cell surfaces are insufficiently close for adhesive or other interactions to occur between them. Alternatively, the nature of the contacting surfaces may be such as to prevent adhesive interactions altogether. For example, the upper surface of the underlapping cell may be nonadhesive (22).

Another situation in which adhesions do not appear to form occurs during contacts between nonlocomotory portions of the surfaces of two cells, as when two cells slide past one another or when the trailing cell body of an underlapping cell comes into contact with the stretched margin of the cell being underlapped. The cells are close enough to affect each other mechanically, as shown by the mutual deformations produced in the cells as they slide past one another, but the cell surfaces freely move past each other apparently without adhering. In these cases it may be that nonmarginal cell surfaces are simply incapable of forming adhesions, either because they are nonadhesive (22) or because they cannot come close enough for adhesion to occur, owing perhaps to electrostatic repulsions. In terms of the latter possibility, Curtis (19) and Weiss (54) have pointed out the difficulties of bringing two planar cell surfaces together. Pethica (42) has suggested that adhesion formation may require that contact be made by cell surface projections of low radius of curvature. Such projections may be lacking on nonmarginal cell surfaces. Of course, another possibility is that weak adhesions do form but are not seen in the light microscope because of phase halo effects. These adhesions may break as the cells move past one another and, thus, may not interfere with cell translocation.

When cell contact does result in the formation of adhesions and in the inhibition of locomotion, often only one of the two cells is inhibited. One cell may continue to extend its margin and underlap the other. Similar examples of this can be seen in thin-section electron micrographs of cells that have contacted and adhered (29, pp. 190–191; 30). The significance of this phenomenon of one-way contact inhibition is not clear, but future attempts to understand it may help to explain the mechanisms of contact inhibition.

The Influence of Cell Shape on the Outcome of Cell Contact Events

If both 3T3 and Py3T3 cells are sensitive to contact inhibition, why then are Py3T3 cells better able to avoid it? The answer appears to be that the morphology of Py3T3 cells and the pattern of their adhesion to the substratum are such as to reduce the probability that contacts between cells will be close enough to result in contact inhibition. Py3T3 cells have stellate shapes with long, narrow processes, and extensive regions of their margins are free from adhesions to the substratum. Locomotory activity is confined to the tips of the narrow processes or to the leading edges of narrow lamellipodia. The geometry of this situation clearly favors underlapping—cells or cell processes with narrow locomotory margins have a high probability of encountering wide marginal areas of other cells that are free of adhesions to the substratum. In 3T3 cell cultures, on the other hand, the geometry discourages underlapping and favors contact inhi-

bition. The overall polygonal morphology of these cells reflects the presence of numerous marginal adhesions to the substratum (28), which often occur in long, apparently continuous bands and which may occupy up to two-thirds of the cell periphery. Thus, 3T3 cells have considerably fewer regions of their margins that are adhesion-free and even these tend to be narrow. The locomotory margins, in contrast, are broad so that contact between 3T3 cells occurs more often between the locomotory margin of one cell and either a locomotory margin or substratum adhesion site of the other cell. In either case, underlapping is usually prevented. 3T3 cells are able to avoid contact inhibition and underlap only when a moving cell encounters a sufficiently wide adhesion-free area of another cell, although the chance that this will occur is less than with Py3T3 cells.

The quantitative data on cell-substratum adhesions and contact events support this interpretation of cell behavior. Py3T3 cell contact events result in underlapping twice as often as those of 3T3 cells, and this overall difference is owing solely to those events occurring between the locomotory margin (lm) of one cell and the inactive margins (im) of another. Inasmuch as locomotory margins are sites of substratum adhesions, all of the nonadhering areas must be along inactive margins. In these lm-to-im contacts, Py3T3 cells, which have a 78% incidence of adhesion-free margin, underlap with a frequency of 85%, whereas 3T3 cells, with only 50% of their margins adhesion-free, underlap only 46% of the time. An increased frequency of underlapping is clearly correlated with a reduced frequency of substratum adhesions, further supporting the contention that the occurrence of contact inhibition is determined by the probability of apparent contacts being close enough to produce an interaction between the cells.

Influence of Cell Shape on Culture Pattern

Although the most important contribution of cell shape to the culture pattern is in influencing the outcome of cell contact events, cell shape can also affect culture patterns directly. A case in point is the difference between the monolayered appearance of highly spread polygonal 3T3 cells and the obvious multilayered appearance of bipolar or stellate Py3T3 cells. In fact, both cell types form multilayers, but because the Py3T3 cells are stellate in shape, when they multilayer they form dramatic crisscrossing patterns that visually emphasize the multilayering. In contrast, the morphology of 3T3 cells actually obscures the multilayering that occurs.

Cell shape also can influence nuclear overlapping, which is often used as a measure of the randomness of cell distribution and hence as an assay for contact inhibition (6–8, 18, 20, 39–41, 56, 57). The underlying assumption of this method is that the nuclear distribution is an accurate reflection of cell distribution; however, this may not always be the case. The nuclear regions of cells tend to be thicker, forming a mound. Because of this, when one cell moves under another, the movement of the nuclear region of the underlapping cell will often be impeded, and the nuclei of the two cells will exclude each other from occupying the same place on the substratum; they simply slide past one another (see Fig. 9). In addition, the mound of cytoplasm usually surrounding the nucleus may keep the nuclei sufficiently separated so that they will appear not even to touch tangentially. As a result, the observed number of nuclear overlaps will be an *underestimate,* even a large underestimate, of the actual amount of cell overlapping. Time-lapse films show that this is precisely what happens in 3T3 cells, which form cytoplasmic multilayers but remain as nuclear monolayers. Py3T3 cell nuclei may be similarly affected during underlapping, although apparently much less frequently.

Role of Cell Division in the Formation of Culture Pattern

A final factor that plays a significant role in the formation of cell patterns in culture is cell division. Because Py3T3 cells have not been observed to crawl over each other, their ability to form dense multilayers is certainly the result of their continued division after confluence is reached. Py3T3 cells may come to lie on the tops of other cells after mitosis, where they remain rounded, but, as long as there is available bare substratum, they eventually move off onto it and spread (22). After confluence, however, the cells begin to pile up, forming dense multilayers which obscure the patterns of crisscrossing formed at low density. 3T3 cells, in contrast, stop dividing soon after they become confluent (49) and, as a result, never produce dense multilayers. However, 3T3 cells can be induced to continue dividing to form dense multilayers, simply by increasing the serum concentration and frequently changing the medium (31), as is true for a number of other types of

monolayering cells (34). Under these circumstances, cell movement will be minimal owing to the crowding of cells. Hence, it is continued cell division and not cell movement that is the principal factor creating the culture pattern. In light of the finding that Py3T3 cells can form multilayers even though they fail to crawl over each other, more attention needs to be paid to the contribution of cell division per se to pattern formation both in vitro and in vivo.

Contact Inhibition and Invasiveness

As discussed earlier, it has been hypothesized that the invasiveness of malignant cells in vivo results from a loss or reduction of contact inhibition (7, 14, 48). The results reported here on Py3T3 cells call this hypothesis into question and suggest the following alternative: that invasion results not by cells crawling over each other, but by infiltration along available noncellular substrata. This hypothesis was first proposed by Barski and Belehradek (9–11) on the basis of their observations of normal cells confronted with tumor cells in monolayer cultures. These malignant cells showed contact inhibition but were able to invade the normal cell outgrowths by working their way through gaps between the cells. My observations on Py3T3 cells are entirely consistent with these observations. Py3T3 cells avoid being contact-inhibited by "invading" the glass or plastic substratum under other cells. Additional support for this hypothesis is provided by observations of DiPasquale and Bell (22). We found that Py3T3 cells, chick heart fibroblast, and S-180 cells, although unable to migrate over the surfaces of epithelial sheets in culture, were able to invade the substratum under the epithelial sheets where the sheet was stretched and not adhering to the substratum. Significantly, S-180 cells invaded less readily than the other cell types, even though their locomotory activity was not inhibited by contact with the marginal cells of the epithelial sheets. Possibly, the more rounded S-180 cells are too thick and therefore are impeded mechanically from underlapping the cell sheets.

Experimental studies of cell invasion in three-dimensional in vitro systems have also indicated that tumor cells invade by infiltrating between other cells. In so doing, they probably move by adhering to a noncellular substratum and not by crawling over other cells. Leighton (35, 36) found that tumor cells invade cells in a sponge matrix culture along the long axis of the fibroblast growth but are blocked by fibroblasts arranged at right angles to the path of invasion. Similarly, Wolff and Schneider (59) reported that sarcoma cells infiltrate fragments of chick embryonic organs along certain preferred routes located in the connective tissue partitions of the organs. These routes are either through cell-free spaces or through tissues with loosely arranged cells, and they all contain noncellular material, including collagen, which the invading cells could be expected to utilize as a locomotory substratum.

The relevance of all of these observations to in vivo invasion is emphasized by their consistency with the observations of pathologists that invading cells follow so-called "lines of least resistance" through organs and tissues. Willis (58) lists the following common paths of tumor invasion in human patients: tissue spaces, intracellular paths, lymph vessels, veins and capillaries, arteries, celomic spaces, cerebrospinal spaces, and epithelial cavities. With the exception of the second, which is a form of emperipolesis, these are all paths that could provide noncellular substrata for invading cells.

In light of all these observations, it seems possible that invasive behavior, whether in model in vitro systems or in vivo, is not necessarily the result of a loss or decrease of contact inhibition. Instead, it may be the result of cells avoiding each other by migrating along preexisting pathways composed of noncellular material. In any event, it is clear that the whole question of contact inhibition of cell movement and its relationship to cell invasiveness needs to be reexamined. In particular, it will be important to consider the role played by noncellular elements—those substances that could provide cells with a substratum for movement and enable them to avoid contact inhibition (38).

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