CONTACT BEHAVIOUR AND PATTERN FORMATION OF BHK AND POLYOMA VIRUS-TRANSFORMED BHK FIBROBLASTS IN CULTURE

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SUMMARY

Certain behavioural and morphological aspects of cellular transformation have been studied, using baby hamster kidney cells (BHK21/13) and polyoma virus-transformed BHK cells. BHK cells grow to monolayers arranged in parallel arrays, whereas the transformed cells show a much greater incidence of crisscrossing and multilayering. Time-lapse cinemicrography and scanning electron microscopy were used to examine the behaviour producing these striking differences in cellular pattern.

It was found, contrary to previous thought, that in both cell lines, when contact is made ruffle to ruffle, ruffling is inhibited. When BHK cells contact each other ruffle to side, strong adhesions always occur, as evidenced by a large deformation of the contacted cell margin, with accompanying paralysis of ruffling. Then, the contacting cell either changes direction, usually spreading along the side of the contacted cell, or occasionally continues to protrude and underlap the other cell, although the original adhesions are seen to remain. Transformed cells never form strong ruffle-to-side adhesions, and usually underlap each other totally. When the cells were filmed, fixed and the same cells relocated in the scanning electron microscope, neither cell type was seen to move over the surface of another (overlap). Rather, all cells criss-cross by underlapping (moving under the other cell). SEM also reveals PyBHK cells to have many fewer side-to-substratum adhesions than BHK cells. The smaller number of these attachments could explain the ease with which PyBHK cells underlap.

INTRODUCTION

Cells derived from tumours usually appear loosely associated in culture and form random, crisscrossed patterns at high density (Ludford, 1934; Lewis, 1936). They also display an increased propensity to divide, even after reaching confluency, so that dense multilayers are produced. Cultures derived from normal tissue, in contrast, generally form a single layer of tightly associated cells on a plasma clot or glass substratum (Loeb, 1921). Although Loeb (1921) observed that cells inhibit each other's movement when they contact each other, it was the statistical analysis of the interaction of primary chick heart fibroblasts by Abercrombie & Heaysman (1953, 1954) which first explained this monolayering phenomenon. Simply stated, cells interfere with each other's movement when they make mutual contact and are prevented from moving in the direction of contact. Cessation of ruffling activity in the direction of locomotion also occurs when the cells contact each other (Abercrombie & Ambrose,

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As a result of this behaviour, ‘normal’ cells become arranged in a ‘monolayer’. It was later shown (Abercrombie, Heaysman & Karthauser, 1957; Abercrombie & Ambrose, 1958) when sarcoma 180 cells and chick heart fibroblasts contact each other, the tumour cells move over the normal cells without impediment or cessation of ruffling. Since tumour cells generate a crisscrossed and multilayered pattern in culture, as do cells transformed by virus (Manaker & Groupé, 1956; Temin & Rubin, 1958; Sachs & Medina, 1961), by chemicals (Bewald & Sachs, 1965), and X-rays (Borek & Sachs, 1966), it has been widely assumed that they are released from contact inhibition of movement and are able to use the upper surface of each other as a substratum for movement. Because of this, changes in growth and morphology of cells in culture came to be used as an assay for transformation, frequently with the assumption that a more crisscrossed pattern signified movement of cells over each other (Temin & Rubin, 1958; Stoker, 1964; Rubin, 1962).

Several largely neglected papers, however, provide evidence that transformed cells, may, in fact, be inhibited by contact with normal cells in vitro. Stoker (1964) observed that when polyoma virus-transformed BHK fibroblasts (PyBHK) were seeded onto a BHK cell monolayer that only covered one half of the culture dish, the PyBHK cells spread to the side of the BHK colony, become oriented in parallel with the cells in the BHK colony but do not move over them. PyBHK cells found elsewhere on the plastic substratum are crisscrossed in irregular patterns. In addition, when PyBHK cells labelled with carbon particles were seeded onto a confluent mouse fibroblast monolayer, the cells rapidly become oriented so that they align with the mouse cells. PyBHK cells which landed on bare plastic become crisscrossed and pile up. These observations led Stoker to suggest that transformed cells are still sensitive to contact with normal cells.

Similarly, when normal mouse embryo heart cells and malignant N1 and M6 strain cells confront each other (Barski & Belehradek, 1965) transformed cells only penetrated the compact normal sheets when gaps open between the cells of the sheet. This local resistance to tumour cell invasion shows that tumour cells do not invariably use the surface of normal cells as a substratum for their movement in culture. KB cells derived from a human epidermoid carcinoma behave in a similar way when co-cultured with normal human kidney amnion cultures (Barski & Belehradek, 1968).

Two recent reports providing detailed time-lapse analysis show that certain transformed cells are, in fact, contact inhibiting according to a number of criteria. Bell (1975, 1977) observed that 3T3 cells and Py3T3 cells both crisscross exclusively by underlapping; however, Py3T3 cells crisscross more frequently, possibly because they have fewer adhesions to the substratum and hence have fewer obstructions to their movement. In addition, he showed that the transformed cells exhibit contact inhibition of ruffling when two fluctuating margins meet. Guelstein et al. (1973) similarly found that there is little overlapping, either in normal mouse or transformed mouse cell cultures, and that underlapping occurs at the same frequency in cultures of both cell types. Normal cells underlap to a small degree and then retract, while transformed cells underlap extensively and often completely crisscross. Thus the
normal and transformed cells differ in the extent to which they underlap but not in
the frequency. Guelstein et al. (1973) apparently did not observe ruffling behaviour.

It appears that direct, detailed observations of contact behaviour between normal
and transformed cells are few and contradictory. If we are to be in a position to general-
ize and resolve the contradictions, further investigations of more transformed cell
lines will be necessary.

The cell clones that I have chosen for such an investigation are BHK21/13 and a
polyoma virus-transformed BHK21 cell line. BHK cells form monolayered parallel
arrays at confluency, while the transformed cells are highly crisscrossed and continue
to divide and multilayer well past confluency (Stoker & Macpherson, 1964). A detailed
analysis of the contact behaviour of these two cell lines was undertaken, using high-
resolution, time-lapse cinemicrography, combined with the SEM, to elucidate the
behavioural parameters which determine their radically different culture patterns.

MATERIALS AND METHODS

Cell culture

Baby hamster kidney fibroblast (BHK21/13) and polyoma virus-transformed BHK21
fibroblast cell lines, originally established by Stoker & Macpherson (1964), were used in these
studies. Both cell lines were a gift of R. D. Goldman. A mycoplasma-free BHK21 line was also
obtained from the American Type Culture Collection (cat. no. CCL10). Upon receipt, 20 am-
poules of each cell type were frozen over liquid nitrogen and stored in a Union Carbide gas-
phase nitrogen freezer. To avoid spontaneous transformation of the cell lines, fresh ampoules
of frozen cells were defrosted for use every 4 weeks. The cells were grown in BHK21 medium
(Gibco), supplemented with 10% calf serum, 10% tryptose phosphate broth, and 1-0% antbiotic-antimycotic solution (Gibco), in 250-ml Falcon flasks (3024) in a 5% CO2 water-
saturated atmosphere at 37 °C. Cultures were transferred every 4 days by removing the cells
from the substratum with Viokase (Gibco, 10X; diluted 1:10 in double-distilled water),
dividing the contents of the flask, and replacing at a lower density in fresh medium.

Time-lapse cinemicrography

Time-lapse films were taken with either a Bolex 16-mm movie camera equipped with a Sage
intervalometer or an Arriflex 16-mm camera and intervalometer. The cameras were used inter-
changeably with a Zeiss Universal microscope, Zeiss inverted D microscope, both equipped
with phase and Nomarski optics, and a Nikon inverted phase-contrast microscope. Plus-X
reversal film 7276 (Kodak) was routinely used at a 0.5-s exposure. All filming took place in a
constant temperature room maintained at 37 °C.

For filming at low magnification, cells were grown in 35-mm Falcon Petri dishes (3001) and
observed with one of the inverted microscopes. To maintain the proper pH, 5% CO2 was
bubbled through water into a plastic hood which fit over the microscope condenser and sealed
around the culture.

In order to observe cells at the highest possible magnification and optimal resolution, a
specially designed filming chamber was used with the Zeiss Universal phase microscope. This
chamber consisted of a 7.5 x 4.5 cm glass slide (Bellco) with a 22-mm hole bored through the
centre. A ring of silicone stopcock grease (Dow Corning) was applied around the hole on both
sides of the slide, and an acid-washed coverslip firmly pressed against one side over the hole.
This assembly was placed in a large Petri dish and autoclaved. Just prior to use, the shallow
well was filled with warm medium and a second coverslip, on which the cells to be observed
were growing, was inverted over the opening and pressed against the slide, thereby sealing the
chamber. This whole assembly was less than 2 mm thick, allowing both condenser and objective
to be oiled to the coverslip. When properly sealed, cultures could be maintained in this
manner for at least 24 h.
In order to film contact events between normal and transformed cells, one cell type was plated on a glass coverslip as usual. Shortly before filming, a suspension of the second cell type was pipetted into the well of the filming chamber. The coverslip with spread cells was inverted over the well, and the chamber placed upright on the stage of the inverted microscope so that the freshly seeded cell suspension would settle on to the coverslip with the spread cells. Filming was begun immediately, so that a record was made of which cells settled to the substratum. One could then differentiate between the 2 cell types in a particular sequence by running the film backward.

All films were analysed with a Kodak L and W Optical Data projector. Contact events were divided into 2 categories: (1) those between the ruffled lamellipodia of 2 cells, and (2) those between the ruffling margin of 1 cell and the lateral non-ruffling margin of another. In both cases, the outcome of the contact event was followed to see whether contact resulted in adhesions, paralysis of ruffling, cessation of forward protrusion, and contact retraction. In addition, it was always noted whether the contacting cell continued to protrude forward or changed direction, this being one of the classic distinctions between contact- and non-contact-inhibited cells. Analysis of heterotypic contacts was the same as for isotypic contacts.

Three methods were used to determine whether cells crisscrossed each other by overlapping or underlapping: (1) Careful focusing at high magnification could sometimes identify which of 2 crisscrossing cells was closer to the substratum. Unfortunately, many cases were equivocal. (2) Alternatively one can observe cell deformations during crisscrossing. As 1 cell moves under another, the movement of its nucleus is often impeded by the margin of the upper cell, slowing its forward movement and, at the same time, deforming the cell above it. When the nucleus finally passes under this barrier, it suddenly pops forward. Had the cell in question been overlapping the other, its nuclear movement would have been constant. (3) A more laborious, but completely unambiguous method, is to film cells and then fix them and prepare them for scanning electron microscopy. Filming indicates which cell had approached and crossed the other, and the SEM provides high resolution and wide depth of field images, which clearly show the relative positions of the cells.

Scanning electron microscopy

Specimens for scanning electron microscopy were prepared according to Porter, Kelley & Andrews (1972), with the following modifications. Cells were plated on acid-washed sterilized 22 x 11 mm glass coverslips. Before fixation, the cells were washed with warm Puck's saline G (Gibco) and fixed for 15 min at 37 °C in 3 % glutaraldehyde, buffered with 0-05 M sodium cacodylate, and 0-5 x Puck's saline to pH 7-2. Cells could be stored at room temperature for several days at this point with no ill effects. After 20 min postfixation with 2 % osmium tetroxide, buffered with 0-2 M cacodylate, the cells were dehydrated through either a graded acetone or ethanol series and critical-point dried in a Sorval critical point drier with liquid CO2, according to the method of Anderson (1951). The cells were then coated with 20 nm of gold, using a rotary shadower (Ladd). Specimens were observed with an ETEC scanner operating at 10 kV.

Some cells were filmed prior to fixation, in order to observe cells in the SEM whose behaviour at the time of fixation had been recorded. The cells to be filmed were cultured on glass coverslips that had been attached to Falcon Petri dishes with silicone grease. When they were engaged in an activity of interest, the medium was carefully removed and the cells were flooded with 3 % glutaraldehyde. The dishes were then marked below the filmed cells with an ink slide marker (Fullum), and the coverslips, in turn, scored with a diamond pencil on the upper surface around the cells concerned. After osmication, dehydration, critical-point drying, and shadowing, the cells were located in the SEM.

Nuclear overlap

In order to quantitate the degree of cell crisscrossing in a culture, the nuclear overlap index, first derived by Abercrombie & Heaysman (1954), and later modified by Weston & Hendricks (1972), was employed. The nuclear overlap index, \( R = O/E \) (where \( O \) = observed number of overlaps and \( E \) = expected number of overlaps), should equal 1, if the nuclei are arranged at random, and should be less than 1 if contact inhibition or some other mechanism prevents cells from moving randomly. The procedure was to select a field of cells in a fixed and stained...
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culture and count the observed number of overlaps. A 40× objective was used, which gave a field area of 4.9×10^4 μm². Fields chosen contained approximately 60 cells.

To compute the expected number of overlaps (E), the average nuclear area first had to be estimated using a Chalkly grid of 25 random points. The graticule was placed in the microscope eyepiece and each point which hit on a nucleus scored. The graticule was then rotated a quarter turn and hits scored again. This process was repeated twice more until the graticule had rotated 360°. The points were then used in the formula for the expected number of overlaps calculated from the nuclear area: \( E = 2a(n-1)/b \), where \( a \) = number of hits on nuclei by random points on the graticule, \( b \) = total number of graticule points projected on the field (in this case 100), and \( n \) = number of cells in the field. Twenty-one fields of BHK cells and 25 of PyBHK cells were analysed in this way.

Table 1. Nuclear overlap index*

<table>
<thead>
<tr>
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<th>PyBHK cells</th>
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<td>N.O.</td>
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<td>0.68</td>
</tr>
<tr>
<td>S</td>
<td>0.04</td>
<td>0.32</td>
</tr>
<tr>
<td>n</td>
<td>21 fields</td>
<td>21 fields</td>
</tr>
</tbody>
</table>

* See Materials and methods (p. 56) for an explanation of this method.

RESULTS

Culture patterns

The differences in culture patterns generated by BHK cells and PyBHK cells are striking. At low density, BHK fibroblasts appear to be uniformly distributed on the substratum (Fig. 1A). At high density, the cells are generally organized in monolayered parallel arrays, similar to those of foetal lung fibroblasts described by Elsdale & Bard (1972; Fig. 1B). If these ‘monolayered’ living cultures are scrutinized at high magnification, however, considerable cellular overlap becomes evident especially in their marginal regions, but there is little crisscrossing of whole cells. This observation is confirmed by examining the cultures in the SEM (Fig. 2). However, unlike their margins, BHK cell nuclei are rarely superimposed and the nuclear overlap index deviates significantly from random (Table 1), i.e. the overlapping of nuclei is much less frequent than would be expected from a random distribution of cells. The nuclei are, therefore, effectively arranged in a monolayer.

Culture patterns generated by PyBHK cells are quite different from those of BHK cells. At low density, PyBHK cells are clumped in small islands (Fig. 1C). It is difficult to ascertain in the phase-contrast microscope if the cells in these clumps are crisscrossed, since the individual cells are quite thick and their margins are obscured by phase halos. SEM micrographs indicate, however, that these clumped cells are indeed crisscrossed (Fig. 3A). This crisscrossing is not as dramatic as at high cell density, however, apparently since the transformed cells have not yet acquired long interlacing processes. At high density, PyBHK cells are multipolar in morphology and their processes are highly crisscrossed (Fig. 1D). This interlacing of cell margins is clearly seen in the scanning electron microscope (Fig. 3C, D). Unlike BHK cells, the nuclei of PyBHK cells are also frequently superimposed, which is reflected in their high nuclear overlap index (Table 1). However, the number of nuclear overlaps
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observed is still less than would be expected if the cells were able to move at random (i.e. N.O. = 1). In addition to being highly crisscrossed, these cells are capable of multiplying well past confluency, with several layers of rounded cells adhering to the upper surface of the spread culture beneath. This multilayering is particularly well visualized in the scanning micrographs of dense PyBHK cultures (Fig. 3c, d).

Contact behaviour

The formation of these culture patterns was observed by filming the cells continuously for 24 h after seeding. When filming BHK cultures at low magnification, one observes that the cells gradually become monolayered in parallel arrays after a series of contact interactions (Fig. 4). When one BHK cell contacts the non-spreading margin of another, formation of adhesions and localized inhibition of ruffling invariably follows. If the angle of contact between the 2 cells is small, the contacting cell alters its direction, so that it comes to lie parallel to the contacted cell (e.g. between cells c and d and between cells d and e in Fig. 4). Thus the direction of movement is modified by contact. It is interesting to note that part of the margin of cell c still continues to ruffle after contact with cell d, and it is from this portion of the margin that forward movement will continue. Once several BHK cells become aligned, they will often move together, always maintaining this aligned configuration. If the angle of contact is large, however, crisscrossing results to varying degrees (e.g. cells a and c in Fig. 9). It is important to emphasize that crisscrossing is observed in cultures of normal BHK cells, but only at low frequency. (The effect of angle of contact upon the outcome of contact events is the subject of another paper in press, Expl Cell Res.)

When 2 BHK cells do crisscross, their nuclei are rarely seen to overlap each other; indeed, this nuclear exclusion is very characteristic of BHK cells and is reflected in their low nuclear overlap index. These nuclear regions appear to be too thick to be superimposed; nuclei are often observed to displace each other and squeeze past each other with considerable strain (Fig. 5).

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Fig. 1. Phase-contrast micrographs of BHK and PyBHK cells in culture. x 220, scale bar = 100 μm.

a. This micrograph shows that BHK cells at low density (400 cells/mm²), 24 h after culturing, are not as elongate as they are at confluency, and generally have 3 or 4 processes rather than the 2 characteristic of bipolar cells. The cytoplasmic areas are quite light, nuclei are distinct, and the bright phase halos around the margins are narrow, indicating that the cells are relatively thin and highly flattened on the substratum.

b. BHK cells at high density (2 x 10³ cells/mm²), after 48 h in culture, become more elongate and bipolar and line up in parallel arrays. There is some crisscrossing, however, especially in the upper right, where an array extending from 1 o’clock to 7 o’clock contacts another at an angle of about 75°.

c. PyBHK cells at low density (400 cells/mm²), 24 h after culturing, are bulbous and multipolar and are generally found in small clumps. Their phase-dark cytoplasm often obscures the nuclei and frequent phase rings indicate that they are less flattened than BHK cells.

d. At high density (2 x 10³ cells/mm²), after 48 h in culture, some PyBHK cells have longer processes and most are obviously crisscrossed. Certain areas of the culture, such as in lower left, are clearly multilayered.
The contact behaviour of PyBHK cells differs in several important respects from that of BHK cells. When a transformed cell appears to contact the margin of another, either (1) the movement of the contacting cell is impeded and the cell will consequently change its direction (as is the case with BHK cells), or, more often, (2) the cells crisscross, usually without an obvious adhesion forming between them, although this was not always established unambiguously in low-magnification films. PyBHK cells clearly crisscross more readily than BHK cells. In fact, even crisscrossing of the
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thick nuclear regions of PyBHK cells is not uncommon (Fig. 6). This results in a much higher nuclear overlap index (Table 1). PyBHK cells continue to crisscross, even at very high densities, and, as a consequence, the cells become extensively entangled.

The mechanism of the clumping of PyBHK cells is also well demonstrated in films. PyBHK cells divide rapidly and move slowly. Thus the rate of increase in number is greater than the rate of emigration from a cluster resulting from cell division. In 10 films analysed, it was found that many more cells are added to a cluster by cell division than leave it by emigration (Table 2).

Active movement of PyBHK cells does not always have to take place for this clumping and crisscrossing to occur, however. Very often several spread cells adhering to each other detach from the substratum and retract, with the result that the cells pile up. Also, multilayers many cells deep are found at high cell density, with rounded cells adhering to the tops of the spread cells (Fig. 3c). From time-lapse films, we know these rounded cells are not mitotic, since they completed cytokinesis many hours earlier; rather they have progressed through mitosis and never respread on the plastic substratum, presumably because of intervening cells beneath (DiPasquale & Bell, 1974; Elsdale & Bard, 1974; Vasiliev et al. 1975). Division in transformed cultures can continue to a point where these cells are even sloughed off into the medium and remain living in suspension.

Underlapping versus overlapping

It is clear that transformed BHK fibroblasts crisscross and form multilayers more extensively than their normal counterparts. This is due to: (1) crisscrossing during active migration, (2) detachment and retraction, and (3) division past confluency.

Abercrombie & Ambrose (1958) showed that sarcoma 180 cells crisscross fibroblasts Fig. 2. Scanning electron micrographs of BHK cells showing alignment and lateral adhesions to the substratum. Scale bars = 10 μm.

a. A SEM of several BHK cells at low density (100 cells/mm²) after 12 h in culture. The cells are not as elongate as at high density (see b) and are more spread laterally. Note that they are very flattened on the substratum and their upper surfaces are quite smooth. These cells already have many points of adhesion to the substratum (arrowheads). × 1000.

b. A low-magnification view of a culture of semi-confluent BHK cells after 48 h in culture. Even though the cells are arranged in parallel arrays, it is clear that there is a good deal more cytoplasmic overlapping than is apparent in phase micrographs. × 500.

c. A confluent BHK cell culture. Note how flattened the cells are and how closely they are apposed to the substratum. The cells also possess many attachments to each other. × 1000.

d. BHK cells are seen here to possess many points of adhesion to the substratum and to each other. Some of these adhesions to the substratum are marked by arrowheads, but note that a few have been torn from the cell during critical point drying. × 780.

e. A high-magnification micrograph of 5 aligned BHK cells. Note how closely the cells are apposed and the many points of adhesion (arrowheads) which are found along the length of their margins. × 3000.

5
Fig. 3. Scanning electron micrographs of PyBHK cells, showing crisscrossing and multilayering. Scale bars = 10 μm.

a. SEM of a clump of PyBHK cells at low density (250 cells/mm²). The cells are crisscrossed and piled up. Note also that their surfaces are covered with blebs and microvilli. Some of the cells near the edge of the clump possess many points of adhesion to the substratum (arrowheads). These adhesions are generally lost when the cells become more spread at high density (compare with b and c). × 1000.

b. Many areas of the cell margins are apparently free of adhesions to the substratum (arrowheads). Note that many of the cells appear to arch over each other when they are crisscrossed rather than make close contact. × 500.

c. Low-magnification view of a multilayered PyBHK culture. The cells that are fully spread on the substratum are highly crisscrossed and multilayered. Note that there are many rounded cells resting on top of the spread cells. × 400.

d. Higher magnification of the boxed area in c. This micrograph emphasizes the interlacing of the many long processes of PyBHK cells. × 1700.
Fig. 4. The formation of parallel arrays by BHK cells. × 340, scale bar = 50 μm. Time elapsed given in min immediately after identifying letter.

A. 0. A BHK culture at the initiation of filming.

B. 60. The same culture 60 min later. Several contact events will occur which will result in the formation of parallel arrays. Cells a and b contact each other’s active margins and cease ruffling (arrowhead). Cell c contacts d at an angle of 30° and localized inhibition of ruffling also occurs (arrowhead). Part of c’s margin below the arrowhead, and not in contact with cell d, is still ruffling.

C. 74. Cell c continues to move with cell d. Parts of its margin not in contact with d are still ruffling (arrowhead). Note how other cells from the lower right are moving in to form a parallel array.

D. 101. Another cell, e, has moved into position parallel to d.

E, F. 114, 137. c, d, e continue their progress forward. Note in E that c and e are still ruffling in areas adjacent to contact with d (arrowheads).

G. 157. Cell f begins to move from its position in frame F, around the curve created by the other cell margins, without visible underlapping. Note that c has now begun to underlap d (arrowhead); e still moves parallel to d.

H, I. 177, 185. f continues to follow a path defined by the margins of the cell above, and f’s ruffling margin is particularly well seen in frame I (arrowhead).
Fig. 5. Movement of BHK cell nuclei during cell crisscrossing. ×550, scale bar = 10 μm. Time elapsed given in min immediately after identifying letter.

A. 0. Cells a and b are already crisscrossed and the nucleus of a is about to pass under b.

B. 12. As the nucleus of a underlaps cell b, it begins to rotate slightly counter-clockwise.

c. 29. The nucleus of a has now rotated almost 45°.

D. 39. Nucleus a continues to move underneath cell b and is still oriented at a 45° angle to the direction of translocation.

E. 41. Nucleus a is now mostly under cell b and the nuclei are not crisscrossing. Nucleus a is clearly being displaced away from the path that it would normally travel. An adhesion between cells a and b is evident at the point marked by the arrowhead.

F. 46. As nucleus a emerges from the other side of b, it rotates clockwise.

g. 48. As cell a moves toward 1 o'clock, it exerts a strong tension on cell b, as seen by the large deformation of b's margin. The nuclei of the 2 cells are at no time superimposed.

H. 59. The nucleus of cell a has now shifted to the right 20 μm, so that it is aligned with the axis of the cell.
Fig. 6. Nuclear overlapping of PyBHK cells. × 340, scale bar = 50 μm. Time elapsed given in min immediately after identifying letter.

A. 0. Cell a has already underlapped cell b with its thin lamella. Note the narrow interlacing processes of the other transformed cells in this field.

B. 47. Half of cell a's nucleus has underlapped b. Note that the leading edge of cell a has emerged from under the other side of b (arrowhead).

C. 55. The 2 nuclei are superimposed.

D. 62. Nucleus a has now underlapped cell b.

Table 2. The dynamics of clump formation by PyBHK cells in culture

<table>
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<tr>
<th>Clump no.</th>
<th>Initial no. of cells in clump</th>
<th>Final no. of cells in clump</th>
<th>Time, min</th>
<th>No. of cells which moved away</th>
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The process of clump formation was studied by counting the number of PyBHK cells added to a clump by mitosis and comparing this figure to the number of cells that migrated away from the clump. Any cell which was not in morphological contact with any other cell in the clump was considered to have migrated away. Note that in any given period of time many more PyBHK cells remain in a cluster than move away.
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by moving over the upper surface of the fibroblasts (overlapping). However, criss-crossing could just as well be due to cells moving under each other (Boyde, Grainger & James, 1969; Weston & Roth, 1969; Harris, 1973; Bell, 1977; see Abercrombie, 1970 for review). If we are to understand the increased occurrence of crisscrossing in transformed cell cultures, we must know by which of these 2 means it is accomplished. As already mentioned (see Materials and methods), there are several ways of distinguishing underlapping and overlapping. The most unequivocal is to film cells and then observe the relative positions of the same cells in the scanning electron microscope. The high resolution and depth of field provided by SEM can establish clearly the relative positions of the cells, while the films preserve a record of how they got there (e.g. Fig. 7). Fifty contact events between polyoma-transformed cells and over a hundred between BHK cells were examined in this fashion. More than twice as many normal cells were observed because they move and contact each other much more frequently than transformed cells. At no time have cells of either type been observed to overlap each other. Rather, after every contact event, the cells that continue to move and crisscross do so by underlapping (see also Figs. 8–10, 12). Thus it was of interest to examine in detail the means by which PyBHK cells underlap each other with great frequency.

Isotypic contacts

A phenomenon associated with contact inhibition is adhesion between the contacting cells and cessation of ruffling. I was, therefore, interested in analysing in detail, at high magnification and high resolution, isotypic contact events, i.e. those that occur between like cells. Contact events were divided into 2 categories: (1) those involving contact between 2 ruffling lamellipodia, and (2) those involving contact between a ruffling lamellipodium and a non-ruffling margin. Contact between ruffling margins of 2 BHK cells invariably results in localized cessation of ruffling of both cells, as predicted (Fig. 8). After initial contact and formation of adhesions, the cells have been observed to interact in 3 ways. (1) One of the margins continues to protrude under the other cell, while the other, now upper, margin remains stationary. No part of the 2 margins that are in contact ruffles after this until the underlapping margin emerges from under the other cell. This protrusion can continue to spread beyond...
the other side of the contacted cell and produce total crisscrossing, or it can protrude just a few micrometres under its neighbour (see Fig. 8). (2) Localized inhibition of ruffling occurs and the cells continue to move by each other by extending uncontacted margins on either side of the cohering region. This allows the 2 cells to shear by each other while still maintaining an adhesion at the original point of contact. (3) The 2 margins may adhere and cease ruffling and, after a time, pull apart (Fig. 8). These cells would then move off in new directions.

The second type of contact between BHK cells, that between the ruffling margin of one cell and the side margin of another, can have either of 2 results. When a ruffling margin collides with the inactive margin of another cell, it usually adheres, ceases ruffling, and, generally, a very strong contraction occurs. The side of the contacted cell is extensively distorted by this contact and contraction and, at the same time, the lateral margin of the contacting cell becomes concave, typical of a cell under tension. This has been termed 'contact retraction' (Abercrombie, Heaysman & Pegrum, 1971; Abercrombie & Dunn, 1975). After adhering, the contacting cell may either; (1) continue to protrude under the other cell and partially or completely underlap it (cells a, c in Fig. 9), or (2) the contacting cell's direction of movement may be modified. The original contact and adhesion is maintained, but that part of the leading edge that is still ruffling shifts direction slightly and leads the cell along the side of the contacted cell. This contact interaction has already been illustrated in Figs. 4 and 6, but is observed at higher magnification and increased resolution in Fig. 9E-H.

Contact events between PyBHK cells were examined in the same manner. When 2 cells approach and contact each other at their leading margins, localized inhibition of ruffling invariably occurs, as between 2 BHK cells. Once contact is established,

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Fig. 8. Details of ruffling activity and contact inhibition of BHK cells. × 1300, scale bar = 10 μm. Time elapsed given in min and immediately after identifying letter.

A. 0:00. A lamellipodium from cell a begins to protrude (arrowhead) toward cell b.
B. 2:40. The ruffling lamellipodium of cell a now reaches, and protrudes over, cell b, as revealed by focusing. At this point, the ruffles of cell a have begun to condense into several phase-dark thickenings.
C. 5:40. Sections of the formerly active margins have ceased moving (small arrowheads). Other parts of the margin have probably not formed adhesions, since they never appeared to contact each other and they still continue to ruffle (large arrowheads).
D. 27:00. Cell a now adheres to cell b along the whole length of its margin. Note that a portion of cell b above the contact region is still ruffling (large arrowheads). A small protrusion from cell b is underlapping a (small arrowheads). The focus here is on the substratum.
E. 32:00. While cell a remains stationary, it is underlapped by cell b (arrowhead). This was identified as underlapping by focusing.
F. 35:00. Both leading edges are now quiescent.
G. 37:00. The two cells begin to pull apart.
H. 45:00. As the cells become almost entirely separated, retraction fibres form (arrowheads), showing how extensive the adhesion areas were. A small portion of cell b is extending under a (large arrowhead). The focus in this frame is on the substratum.
several alternative modes of behaviour are observed. (1) Like BHK cells, one of the margins may remain stationary and the second continues to protrude forward, beneath the other cell (Fig. 10). In this particular sequence, the underlapping margin apparently does not ruffle, possibly because uplift was impeded by the cell above. (2) Since inhibition of ruffling is localized, some parts of one of the active margins may never contact the other cell. Thus the cells may shear past each other, utilizing portions of the margins which are still free to extend. (3) The cells may adhere, with little or no protrusion by the margin of either cell and eventually pull apart, leaving retraction fibres behind as they do so (Fig. 11). From all this, it is obvious that there is no discernible qualitative difference in the behaviour of BHK and PyBHK cells when a ruffling margin of one cell contacts a ruffling margin of another.

When the ruffle of a PyBHK cell meets the side margin of another PyBHK cell (Fig. 12), no obvious adhesions are generated and the cells crisscross by one underlapping the other. To be sure, ruffling stops as the cells crisscross, but this is probably due to mechanical prevention of upward folding by the presence of a cell above rather than to paralysis of the machinery responsible for ruffling. When, in a few instances, adhesions are formed, very little, if any, deformation of the contacted cell is noted. Thus there appears to be no contact retraction. In almost every case where a ruffling margin meets the side of another cell, forward movement continues under the other cell. Total crisscrossing of the 2 cells does not always occur, however. At times

Fig. 9. Localized contact inhibition of ruffling of one BHK cell by the side margin of another. x 900, scale bar = 10 \(\mu\)m. Time elapsed given in min immediately after identifying letter.

A. 0. Cell \textbf{a} contacts cell \textbf{c} with its ruffling lamellipodium (arrow). Note that although \textbf{a} and \textbf{b} are adhering tightly (small arrowheads), both cells still ruffle in the regions where they are not in contact (large arrowheads).

B. 9. Cell \textbf{a} is clearly adhering to \textbf{c}, as evidenced by the deformation of the margin of \textbf{c}. Cell \textbf{b} has not yet contacted \textbf{c}.

C. 12. \textbf{a} is still adhering to \textbf{c} and here has underlapped it slightly (small arrowheads). This was identified as an underlap since it is at the same focal level as the substratum, whereas cell \textbf{c} is not. Note that \textbf{a} is spreading on the substratum at other loci (large arrowhead); \textbf{b} still continues to ruffle (arrowhead) even though it is in close contact with \textbf{a} along its whole length.

D. 17. While \textbf{a} remains adherent to \textbf{c}, \textbf{b} continues to ruffle (arrowhead). Note how drastically the margin of \textbf{c} has been deformed by the tension exerted on it by \textbf{a}. The extent of the underlapping by \textbf{a} is clearly seen here (small arrowheads) since the focus is now on the substratum.

E. 24. \textbf{b} finally underlaps \textbf{c} (large arrowhead), but it is not possible to tell if adhesions were formed with \textbf{c}.

F. 41. \textbf{a} and \textbf{b} still adhere to, and underlap \textbf{c}. Note that another cell, \textbf{e}, which first entered the field in \textbf{D}, has contacted a small ruffling margin of \textbf{a} at an angle of 30°.

G. 44. \textbf{a} and \textbf{e} are seen to be adhering as evidenced by the tiny retraction fibres connecting them (arrowheads). The remainder of \textbf{e}'s margin below the adhesion area is still ruffling.

H. 54. \textbf{a} and \textbf{b} are still apparently underlapping \textbf{c}. \textbf{e} has now moved 10 \(\mu\)m along the margin of \textbf{a} and has a margin ruffling on the side away from its contact with \textbf{a}. Note that \textbf{e} has continued to move parallel to \textbf{a} without underlapping it or moving away into the cell-free space on the other side.
Contact behaviour of BHK and PyBHK cells in culture

Table 3. Summary of isotypic contact events of BHK cells and PyBHK cells

<table>
<thead>
<tr>
<th></th>
<th>BHK cells</th>
<th>PyBHK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total no. of events</strong></td>
<td>76</td>
<td>85</td>
</tr>
<tr>
<td><strong>% resulting in adhesion</strong></td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><strong>No. of contacts</strong></td>
<td>12</td>
<td>27</td>
</tr>
<tr>
<td><strong>ruffle to ruffle</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no. underlapping</td>
<td>3 (25 %)</td>
<td>11 (41 %)</td>
</tr>
<tr>
<td>no. pulling apart</td>
<td>5 (42 %)</td>
<td>9 (33 %)</td>
</tr>
<tr>
<td>no. that move past the adhesion point</td>
<td>4 (33 %)</td>
<td>7 (26 %)</td>
</tr>
<tr>
<td><strong>No. of contacts</strong></td>
<td>64</td>
<td>58</td>
</tr>
<tr>
<td><strong>ruffle to margin</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no. producing a change in direction of movement</td>
<td>41 (64 %)</td>
<td>4 (7 %)</td>
</tr>
<tr>
<td>no. producing an underlap</td>
<td>23 (36 %)</td>
<td>54 (93 %)</td>
</tr>
</tbody>
</table>

Fig. 10. Contact inhibition of ruffling between the lamellipodia of 2 PyBHK cells, and underlapping. × 900, scale bar = 10 μm. Time elapse given in min immediately after identifying letter.

a. o. Cell b approaches cell a.
b. 7. The protruding lamellipodia of cells a and b collide (arrowhead).
c. 10. The margin of cell b protrudes past the margin of cell a. Focusing shows that cell b is underlapping cell a.
d. 12. All signs of ruffling have now ceased at the point of contact of cells a and b, and cell b has retracted slightly, as evidenced by its concave margins.
e. 13. b now begins to protrude under a (arrowheads).
f. 15. The margin of cell b moves very rapidly, travelling the 35-μm distance under a in 8 min. The edge of the process under a is marked by small arrowheads. Focusing indicated that this cell did not ruffle at all while it was under a.
g. 24. Cell b emerges out from under cell a with a burst (arrowhead). Note that this process is not a flattened protrusion, but quite thickened.
h. 31. Once having escaped from under the confines of the cell above, cell b begins to ruffle again (arrowhead).
PyBHK nuclei are seen to exclude each other and the contacting cell is forced to change its direction.

These contact events are summarized in Table 3.

*Heterotypic contacts*

Because of the possible significance of reduced contact inhibition for invasiveness, it was of interest to see if PyBHK cells would move over BHK cells in culture. When PyBHK cells are seeded on BHK monolayers they are usually seen to fill in the gaps between the parallel-arrayed BHK cells and become bipolar themselves (Fig. 13).
Occasionally, PyBHK cells will also crisscross BHK cells. In no case, however, does this crisscrossing occur by overlapping. Rather, all crisscrossing is accomplished by underlapping. When the 2 cell types are mixed, mutual inhibition of ruffling is also observed. That is, when the active margins of a BHK cell and a PyBHK cell meet, there is cohesion and cessation of ruffling (Fig. 13).

**Analysis of underlapping**

We have seen that the crisscrossing and multilayering of PyBHK cells may occur in several ways. At low density they contract into clumps, while at high density they continue to multilayer by cell division. Equally important is the generation of crisscrossed patterns by active underlapping of other cells. Three hypotheses could explain why PyBHK cells underlap each other more than BHK cells.

1. BHK cells may be more tightly apposed to the substratum than PyBHK cells. This idea is supported by the absence of underlapping especially in the thick nuclear regions. It follows that if BHK cell margins are closer to the substratum than PyBHK margins, they might be in a more optimal position to form an adhesive contact with the thin lamellipodium of another cell, since lamellipodia are always closely applied to the substratum.

2. BHK fibroblasts may have many more adhesions to the substratum than PyBHK cells, which might obstruct underlapping. This initially seemed unlikely, however, since Harris (1973) showed that bipolar cells generally possess few adhesions to the substratum and these are confined to the ends of the cells.

3. BHK cells are able to form stable adhesions with each other, whereas PyBHK cells may not.

Clearly one or a combination of these hypotheses might explain the reduced ability of BHK cells to crisscross each other. In order to choose among them, the morphological properties of BHK cells and PyBHK cells were compared.
A description of cell morphology

Monolayered BHK cells are bipolar and very elongate. They appear quite thin and stretched and, in phase-contrast, the phase halos around them are small and their cytoplasmic areas are relatively light, confirming their thinness (Fig. 1A, B).

PyBHK cells, in contrast, are multipolar in morphology (Fig. 1D). At high magnification, the cells appear bulbous and not as flattened as their normal counterparts, as emphasized by their large phase halos and relatively dark cytoplasms.

Fig. 12. Underlapping of PyBHK cell margins by ruffling lamellipodia without formation of adhesions. ×900, scale bar = 10 μm. Time elapsed given in min immediately after identifying letter.

A. 0. PyBHK cell c has just made contact with cell b (arrowhead).
B. 4. Cell b slightly underlaps cell c (arrowhead), apparently without adhering, since the margin of cell c has not been deformed in any way. Note also a large protrusion of cell a (small arrowheads) which fluctuates freely under cell c throughout this sequence. Underlapping was determined by focusing on the substratum.
C. 15. Cell b has now extensively underlapped cell c (arrowheads), still apparently without generating adhesions to cell c. Cell c approaches cell a at points 1 and 2.
D. 25. Cell c has underlapped cell a at points 1 and 2 apparently without adhering, since the margins of cell a are undisturbed.

The observations of BHK cell morphology with phase-contrast microscopy are reinforced and expanded by the SEM. At high density, BHK cells in parallel arrays have accentuated bipolarity and are very elongate. These fibroblasts are practically devoid of surface protrusions, such as microvilli or blebs, when they are fully spread, indicating that they are highly flattened (Erickson & Trinkaus, 1976). Occasionally,
they are observed to have lines of stress or tension running the length of their surfaces. Cells at high density are particularly thin and flattened against the substratum (Fig. 2), and their margins seem quite tightly apposed to the substratum.

PyBHK cells in the SEM are more bulbous and rounded than BHK cells, and at high density do not appear to be as tightly apposed to each other and the substratum as are their normal counterparts (Fig. 3B–D). These cells have microvilli and even maximally spread cells at very high density sometimes have microvilli, although they are quite short. The loose apposition of the margins to the substratum is emphasized by the fact that PyBHK cells overlapping each other do not seem to be tightly apposed. Indeed, the SEM gives the impression that PyBHK cells actually arch over each other (Fig. 3B).

**Table 4. Number of adhesion points per cell**

<table>
<thead>
<tr>
<th></th>
<th>BHK cells</th>
<th>PyBHK cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average no.</td>
<td>27.56</td>
<td>12.23</td>
</tr>
<tr>
<td>±</td>
<td>6.79</td>
<td>6.79</td>
</tr>
<tr>
<td>n</td>
<td>36 cells</td>
<td>30 cells</td>
</tr>
</tbody>
</table>

The no. of adhesion points per cell, as determined from SEM micrographs of confluent cultures and then averaged.

**SEM visualization of adhesion plaques**

The scanning electron microscope provides a more detailed picture of the distribution of adhesions than can be obtained by phase microscopy. One can identify many regions of the margin of BHK cells which are free of contact points to the substratum at low density. Nonetheless, the whole margin appears to be quite closely apposed to the substratum, despite the low number of actual contact points. At high density, in contrast, the majority of BHK cells possess extensive areas of adhesion along their margins (Fig. 2), although they are by no means pinned down around the whole periphery. In addition, they often appear to adhere to each other along their entire length.

At high density, PyBHK cells have few marginal adhesions to the substratum, and possess long, thin processes which are mostly free of substratum attachments, except at the tip (Fig. 3). Intercellular adhesions between PyBHK cells do not appear to be as extensive as those connecting BHK cells.

The number of observed marginal adhesions to the substratum for each cell type is shown in Table 4. This table shows that BHK cells have on the average 3 times as many such adhesions as PyBHK cells.

**Analysis of the significance of the adhesions**

To test the role of cell-substratum adhesions in generating specific culture patterns, BHK cells were plated on a number of substrata of differing adhesiveness. Unfalconized plastic is a lowly adhesive substratum for cells, due, apparently, to its neutral charge. BHK cells spread much less on unfalconized plastic than on Falcon plastic, but are still bipolar in morphology. Crisscrossing increases dramatically, however
Contact behaviour of BHK and PyBHK cells in culture

The SEM reveals these cells to have many fewer marginal adhesions to the substratum (Fig. 14n, c). Although the cells are bipolar, they are not as elongate as on Falconized plastic, and are spindly, possibly since there are not enough adhesions to pin them down laterally. Moreover, they have many more microvilli on their upper surfaces than when spread on Falconized plastic (Fig. 14d), consistent with the idea that they are less spread and flattened (Erickson & Trinkaus, 1976).

Time-lapse films provide 2 explanations for the increase in crisscrossing on unfalconized plastic. First, cells adhering together are often seen to contract into clumps, suggesting that the cells adhere less tightly to the substratum than to each other. Second, they underlap more frequently. This may be due to loss of marginal adhesions to the substratum, which might otherwise have restricted cell crisscrossing.

It seems possible, then, that at least two of the factors originally hypothesized may contribute to the reduced ability of BHK cells to underlap. They have more marginal adhesions to the substratum than PyBHK cells and the margins themselves are quite tightly apposed to the substratum. Whether these points of adhesion serve simply as blockages to cell crisscrossing, or pull the margins closer to the substratum so that contact and consequent contact inhibition can occur more readily, is not known.

DISCUSSION

Contact inhibition of movement

Contact inhibition of movement involves a cessation of ruffling upon contact and a failure of cells to migrate in the direction of contact. This has generally carried with it the implication 'that one cell does not use another as a substratum' (Abercrombie, 1970, p. 138). Since most transformed cells are highly crisscrossed and form multilayers in culture, it has been assumed that they show reduced contact inhibition. BHK fibroblasts form monolayered, parallel arrays in culture, whereas PyBHK cells exhibit a much greater incidence of nuclear and cytoplasmic overlapping, resulting in extensive crisscrossing and multilayering. In the present study, it was found that

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Fig. 13. Alignment and inhibition of ruffling in a mixed co-culture of BHK cells and PyBHK cells. × 600, scale bar = 10 μm. Time elapsed given in min immediately after identifying letter.

A. o. Two minutes after PyBHK cells were seeded onto a 24-h culture of BHK cells, and allowed to spread. The rounded transformed cells a, b, and c have begun to send out processes.

B. 26. Cells a, b, and c have now started to spread along the sides of the BHK cells; d has also begun to ruffle (arrowhead).

C. 36. As spreading continues, the transformed cells align with the normal cells. Cell d has extended its margin, and here makes contact with the ruffling margin of BHK cell e.

D. 40. Ruffling ceases in the area of contact between cells d and e. Note that there is still ruffling activity along the sides of the 2 cells where they have not yet contacted (arrowheads).

E. 41, 67. PyBHK cells still continue to move parallel to BHK cells. Cell d remains adherent to the BHK fibroblast e without ruffling in the region of contact.

F. 98. Note that while cell a is aligned with a BHK cell, its leading free edge is still ruffling (arrowhead).
Fig. 14. BHK cell cultures on unfalconized plastic.
a. BHK cells cultured for 24 h are more narrow and spindly than usual. There is also a tremendous increase in crisscrossing, compared with cultures grown on Falconized plastic (compare to Fig. 1). ×190, scale bar = 100 μm.

b. SEM of BHK cells cultured for 24 h emphasizes the impression from a that the cells are more spindly. ×200, scale bar = 10 μm.

c. SEM of the culture in b. One finds fewer marginal adhesions to the substratum than on Falconized plastic (compare with Fig. 3). Most of the cells are also less flattened and are narrower. ×700, scale bar = 10 μm.

d. SEM of the cell marked by an arrowhead in b. Many more microvilli are observed on the surface of this cell and on most others grown on unfalconized plastic than are seen on typical BHK cells. This correlates with their apparently less-spread condition. ×9000, scale bar = 1 μm.

in both cell lines local paralysis of ruffling occurs when 2 ruffling margins adhere. Moreover, when examined closely, BHK as well as PyBHK cells display a certain degree of crisscrossing and it was found that this crisscrossing in both cell types invariably results from one cell moving under the other. None was observed to move over the upper surface of another cell. Both BHK and PyBHK cells, therefore, show
Contact behaviour of BHK and PyBHK cells in culture

Qualitatively the same behaviour. Contact inhibition was also observed after heterotypic contacts between normal and transformed cells. When ruffling margins of a BHK cell and a PyBHK cell adhere, ruffling and movement cease. Furthermore, BHK and PyBHK cells crisscross each other exclusively by underlapping.

The increased crisscrossing in transformed cultures appears to be based on a quantitative rather than a qualitative difference in contact behaviour between the 2 cell types; PyBHK cells are able to underlap each other more readily.

How, then, are we to account for the increased tendency of PyBHK cells to underlap? It was observed that when the ruffling margin of one BHK cell contacts the side of another, adhesions are generally produced and ruffling activity ceases at the point of contact. The majority of these contact events (64%) result in inhibition of further movement in that direction and the contacting cell will then alter its path of translocation. Polyoma-transformed cells, in contrast, rarely form visible adhesions when a ruffling margin contacts the side of another PyBHK cell; instead, they usually crisscross unimpeded. Similarly, in the rare event where no adhesions are generated between BHK cells, underlapping also proceeds. This underlapping in the apparent absence of adhesion has also been observed by Harris (1973) and Bell (1977) in other cell lines. It appears, therefore, that in general when contacting cells do not adhere, they underlap.

Two other factors that do not involve loss or reduction of contact contribute to multilayering of the PyBHK cells. PyBHK cells are frequently observed to lose their adhesions with the substratum and to retract into clumps, in which the cells are piled up and crisscrossed; and cells may also multilayer by continuous cell division. Although it has been shown that the upper surfaces of fibroblasts and epithelial sheets will not support spreading (DiPasquale & Bell, 1974; Elsdale & Bard, 1974), rounded cells may pile up on the surface of the sheet if they continue to divide. It is clear that in both cases, multilayering occurs without cells using each other as a substratum for locomotion.

Influence of cell morphology on underlapping

Two possibilities could account for the reduced number of adhesions between PyBHK cells and consequently for the frequent movement of these cells under each other. Either transformed cells are deficient in their ability to generate adhesions upon contact, or their margins do not actually come into contact frequently. Observations of cell morphology favour the latter.

Although the ruffling margin of one PyBHK cell may appear in the light microscope to contact the side of another PyBHK cell, the 2 may not be in contact at all. When cells are observed with the light microscope, the view is essentially 2-dimensional, with the critical ‘depth of field’ being severely limited. Thus one cell might be protruding over or under the other and be separated from it to a considerable degree, yet appear to be touching it. Scanning electron micrographs provide evidence on this matter. PyBHK cells have few adhesions to the substratum and consequently their margins are generally lifted off it. Thus, 2 crisscrossing cells are often seen to be clearly separated, with no evident intercellular adhesions. BHK cells, on the other
hand, have many marginal adhesions to the substratum, and their margins, in general, are much more tightly apposed to the substratum. This correlates well with their greater tendency to adhere to each other upon contact and to underlap infrequently. Conversely, when BHK cells lose most of their marginal adhesions on lowly-adhesive unfalconized plastic so that their margins are no longer tightly apposed to the substratum, crisscrossing due to underlapping increases dramatically. This supports the contention that these adhesions and/or the proximity of the margins to the substratum prevent crisscrossing. Unfortunately, it could not be determined whether it is the adhesions themselves which prevent underlapping or simply that these adhesions draw the cell margins closer to the substratum, and that this subsequently inhibits underlapping. It may be that a similar change in the distribution of adhesions occurs after cells are extracted with urea (Weston & Hendricks, 1972) and could account for the resultant increase in crisscrossing under this condition as well.

Of course, we cannot rule out the possibility that PyBHK cells are deficient in their ability to form adhesions to each other, even if their margins do contract. However, there are 2 observations which argue against such a deficiency. First, when contact is made between 2 PyBHK cell margins which are both close to the substratum (i.e. 2 ruffling margins), adhesions always form. Thus, given the optimal juxtaposition of margins, PyBHK cells will adhere and cease ruffling. Second, when PyBHK cells are seeded onto a confluent BHK culture, the transformed cells adhere to the margins of BHK cells, cease ruffling, and even align with the BHK cells.

It appears, therefore, that both of these cell types do exhibit contact inhibition of movement when their surfaces are known to be making contact, especially ruffle-to-ruffle contact. The following observations support the idea that PyBHK cells do not show a loss of contact inhibition: (1) they inhibit each other's ruffling as well as BHK cells' ruffling; (2) they are prevented from continuing to move in one direction after contact with the side of a BHK cell. It is possible that when 2 PyBHK cells criss-cross, they are not in contact. If indeed they do not touch, we should be careful about attributing the resulting culture morphology to a loss or reduction in contact inhibition, since contact inhibition of movement is, by definition, cessation of movement after cells have made contact with other cells.

The fact remains that for at least 2 cell systems examined in detail, BHK cells and 3T3 cells (Bell, 1977), the transformed cells are more crisscrossed because they underlap more frequently and not because they move over each other, as has been shown in one case (Abercrombie & Ambrose, 1958) and inferred in others where crisscrossing is observed (Temin & Rubin, 1958; Bewald & Sachs, 1965; Borek & Sachs, 1966). Whether we judge transformed BHK cells to exhibit contact inhibition or not may be premature at this time, especially since underlapping has not been proven to occur without contact. Until we know precisely the mechanism for contact inhibition, it would be wise to describe the events associated with contact rather than to label them. Thus qualitatively, PyBHK cell behaviour is the same as BHK cell contact behaviour in that they cease ruffling when they approach the ruffled margins of another cell and they become superimposed when 2 cells move under each other rather than utilizing another cell as a substratum for locomotion.
Contact behaviour of BHK and PyBHK cells in culture

General implications

It seems clear, on the basis of this and several other studies where cell behaviour was observed directly (Barski & Belehradek, 1965; Gulestein et al. 1973; Bell, 1977; Vesely & Weiss, 1973), that the concept that transformed cells show reduced contact inhibition of cell locomotion should be reconsidered. Unfortunately, such direct observations of contact behaviour have been infrequent and consequently most conclusions about differences between normal and transformed cells have been drawn from appearances of the patterns of cells in culture after they have already formed and without concern as to how they got there. The evidence that transformed cells do not differ from the normal cells qualitatively in their behaviour after contact in culture is mounting and suggests that other transformed lines should be scrutinized before any generalizations can be made. Indeed the problem demands careful consideration since loss of, or reduction in contact inhibition, may not be a result of transformation and may have little significance for understanding the more important problem of invasiveness of tumour cells in vivo.

I would like to thank my thesis adviser, Dr J. P. Trinkaus, for his support while this work was in progress and his help in preparing the manuscript.

This work was supported by NSF grant BHS 70-00610 and NIH grant USP HS-HD 07137 to J.P.T. and by a fellowship from NIH Training grant HD-00032-11 to C.A.E.

This work represents part of a dissertation submitted to the Graduate School of Yale University in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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(Received 24 October 1977 – Revised 10 March 1978)