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MOVEMENT IN A CONFLUENT 3T3 MONOLAYER AND THE CAUSES OF CONTACT INHIBITION OF OVERLAPPING

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SUMMARY

The existence of contact inhibition of nuclear overlapping (monolayering) has previously been interpreted to mean that intercellular contact locally inhibits the cell's locomotory apparatus and hence that cells in a confluent monolayer should be immobilized. Garrod & Steinberg, however, observed gross movements and exchanging of nuclear nearest neighbour relationships in a confluent monolayer of chick epithelial-like cells. Data presented in the present report reveal that similar movements occur in confluent monolayers of mouse 3T3 fibroblasts, previously shown to display contact inhibition of speed, contact inhibition of overlapping, and postconfluence inhibition of cell division.

Cytoplasmic boundaries frequently could not be resolved in the present study. However, during a 2·2-day period, more than one third of originally adjacent pairs of nuclei became separated by 1–5 intervening nuclei, and their average distance of separation increased more than 2-fold. Also, each nucleus was displaced, on average, more than one mean cell diameter in a random direction. These indications of gross cellular movements were seen during the stationary density phase of perfused culture, as well as during the doubling of cell density which occurs after confluence but before the stationary phase.

If the intensity of cell–substratum adherence exceeds that of cell–cell adherence, this differential in adhesive strength could, by itself, produce the observed avoidance of overlapping, without invoking local inhibition of the cell's internal 'motor' by contact. This differential adhesion hypothesis for contact inhibition of overlapping is consistent with the extensive movements very likely occurring in at least certain confluent monolayers. On the other hand, the probable existence of these movements seems less easily reconciled with the hypothesis that contact inhibition of overlapping results from contact-induced local paralysis of an intracellular locomotory apparatus, although this latter hypothesis is not excluded.

INTRODUCTION

Normal epithelial-like or fibroblast-like cells from certain vertebrate tissues form cell monolayers in culture (Abercrombie & Heaysman, 1954; Todaro, Green & Goldberg, 1964; Abercrombie, Lamont & Stephenson, 1968; Garrod & Steinberg, in preparation; Martz, 1973; for review, see Martz & Steinberg, 1973). The pronounced tendency of these cells to remain in a monolayer (a description at the level of the cell population) may also be described as a tendency of each cell not to move under, over, or upon other cells (a description at the level of pairs of cells). The individual cells are

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motile, and move with varying speeds (Abercrombie & Heaysman, 1954; Martz, 1973) in apparently random directions in the absence of directional influences such as population density gradients or substratum inhomogeneity (Weiss, 1934; Abercrombie & Heaysman, 1953; Rosenberg, 1963; Abercrombie & Gitlin, 1965; Carter, 1965; Abercrombie, 1967). When one cell encounters another, it usually ceases movement along its original (collision) course, and eventually takes up movement in a direction not obstructed by another cell (Loeb, 1921; Abercrombie & Ambrose, 1958; Trinkaus, Betchaku & Krulikowski, 1971). This behaviour was described as 'contact inhibition' of cell movement by Abercrombie & Heaysman (1954), a term fully justified by the demonstration that the avoidance of cell overlapping was the result only of cell–cell contact, and not of diffusible materials concentrated or depleted in the vicinity of cells (Abercrombie & Heaysman, 1954; Abercrombie & Gitlin, 1965; reviewed by Martz & Steinberg, 1973).

Abercrombie & Heaysman (1954) originally reported that 'in films of cultures in which two sheets of cells had joined closely together the cells can be seen to be moving independently, and very occasionally to change their immediate neighbours. The variations in the cell population within a given area which occur after close packing has been attained are also evidence of continued movement.' Despite this observation, the concept of 'contact inhibition' has sometimes led to the supposition that a cell experiencing contact with other cells on all sides would be unable to move in any direction (Abercrombie, Heaysman & Karthauser, 1957; Weston & Abercrombie, 1967). Hence, it has sometimes been assumed that cells in confluent monolayers would be immobilized. It should be noted, however, that the usual operational definition of this form of contact inhibition – namely, fewer nuclear overlaps than expected from random distribution (Abercrombie & Heaysman, 1954) – does not imply that cells in a confluent monolayer need be immobilized. (For this reason, we prefer the operational term 'contact inhibition of overlapping' [Martz & Steinberg, 1973], which is intended to include 'contact inhibition' in the original sense, but to carry no mechanistic connotations.) Moreover, no one, to our knowledge, has reported a case in which cells are immobilized in a confluent monolayer in culture.

On the contrary, the average speed of the nucleus of an isolated chick cell was reduced only 50% by the effects of simultaneous contact with *six* other cells in Abercrombie & Heaysman's (1953) original study. Although this percentage was obtained for a cell population with a net directional movement away from an explant, a similar percentage reduction has been observed in a population of 3T3 mouse fibroblasts with no net population movement (Martz, 1973). The amount of the cell's perimeter in contact with other cells was not measured in the early study of chick cells, but it was measured in the more recent study of the speeds of 3T3 cell nuclei (Martz, 1973). In that study, the effect, on speed, of cell–cell contact measured as amount of *perimeter* in contact with other cells closely paralleled the effect of the *number* of contacting cells. Speed was reduced only about 50% by contact levels of 5–6 or 100% of the cell's perimeter.

Measurements of nuclear speed, however, could conceivably reflect only small oscillations-in-place, and do not necessarily imply that significant distances were

traversed by whole cells. In the first attempt to obtain quantitative data relevant to this question, Garrod & Steinberg (in preparation) examined the movements of chick embryonic liver epithelium-like cells in a confluent monolayer. They found striking movements of individual cell nuclei resulting in much shifting of nearest-neighbour relationships. Data presented in the present communication reveal that similar movements occur in confluent monolayers of mouse fibroblast-like 3T3 cells previously demonstrated to exhibit contact inhibition of overlapping and of speed (Martz, 1973). Preliminary discussion of these results has been made elsewhere (Steinberg, 1973).

MATERIALS AND METHODS

Cultures of the established mouse fibroblast line 3T3 (Todaro & Green, 1963) were filmed in a specially designed, perfused culture chamber (Martz, 1969) according to a time-lapse cinemicrographic protocol previously described (Martz & Steinberg, 1972). The filming was done with Nomarski optics using a 10× objective. The culture medium was Dulbecco-Vogt's modification of Eagle's basal medium, with 3·3% foetal calf serum. One culture, which was filmed continuously during exponential growth and postconfluence inhibition of cell division, including 2·5 days of stationary density phase (total 7·6 days) was the subject of previous reports (Martz & Steinberg, 1972; Martz, 1973). Additional data from the film of this culture (Film 18) are the subject of this report. Data were obtained from the film with the use of an analytical projector (L-W Photo, Inc., Van Nuys, California, Model 224-A).

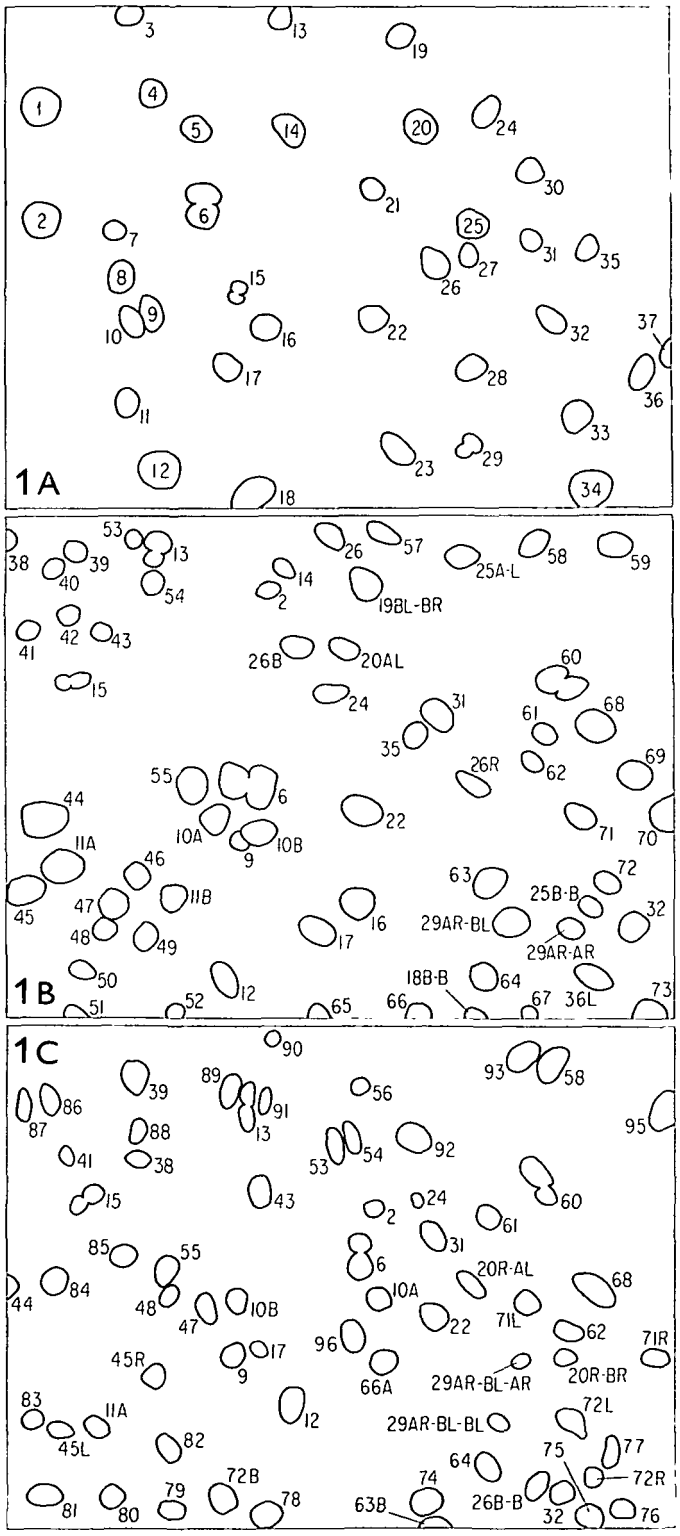
RESULTS

The intervals selected for movement analysis

Two equal time intervals were selected for the analysis of cell movements in the confluent 3T3 monolayer recorded in Film 18. Each interval had a duration of 2·2 days (53 h 20 min). The first interval begins with Frame I (Fig. 1A), when confluency has practically been obtained (one small gap remaining between the cells closes in the first few hours). Frame II (Fig. 1B) is the end of the first interval and the beginning of the second. During the first interval, the number of cells in the photomicrographic field increases from 37 to 64; during the second, the number of cells remains essentially stationary. The second interval ends with Frame III (Fig. 1C), containing 68 cells. There are 29 mitoses during the first interval and 14 during the second, which correspond to mitotic rates of 0·26 and 0·10 mitoses/cell/day, respectively.

Development of objective criteria for neighbour relationships

In order to assess the amount of movement of the cells relative to each other in the confluent monolayer, we wanted to know how many cells adjacent (neighbours) at the beginning of each interval were no longer adjacent by the end of the interval. In other words, we wished to quantitate the average number of neighbours lost and gained (exchanged) around each cell. Initially, an attempt was made to discern which pairs of cells in Frame I were 'adjacent' by scrutinizing the film running forward and backward around this point in time. This proved quite difficult because the cytoplasmic boundaries of the cells became indistinct at confluency, and because the area occupied by different cells varied considerably. (The mid-80% of 3T3 cells from a culture in


1A
1B
1C

the stationary density phase has a 4-fold range in volume [Martz, 1969].) Therefore, objective criteria for assigning neighbour and non-neighbour relationships were devised, as detailed in Fig. 2. These criteria utilize the relative positions of the cell nuclei, since the cytoplasmic boundaries could not be visualized in many cases.

The exact set of rules used to decide whether or not 2 nuclei are neighbours or non-neighbours is necessarily arbitrary. However, since the hypothesis which we wish to test states that there is no relative movement of the cells in a confluent monolayer, it is important that the rules used do not produce an overestimate of the amount of relative movement.

The criteria were based on the principle that if, during a given time interval, 2 initially adjacent cell nuclei became separated by a third intervening nucleus, then the neighbour relationship of the originally adjacent cell nuclei was *lost*. Similarly, if 2 cell nuclei, which were initially separated by an intervening nucleus, became adjacent, each would be considered to have *gained* the other as a new neighbour. Neighbour gains should equal neighbour losses, provided that the average number of neighbours per cell does not change during the time interval studied. Hence the percentage of neighbours lost (or gained) during a given time interval will indicate the amount of relative movement of the cell nuclei.

In order to avoid overestimating the amount of neighbour losses, pairs of nuclei which were neither clearly neighbours nor clearly non-neighbours were assigned an *intermediate* relationship (Fig. 2). Any changes in relationships which began or ended as intermediate were not considered gains or losses. We defined the intermediate category so as to be broad, in order not to overestimate gains and losses.

The results of the objective criteria were compared with the subjective judgments we had made earlier on Frame I. In the subjective examination of 9 cells, we had assigned each cell an average of 4·0 neighbours and 3·7 intermediates. When applied to subjective neighbours, the objective criteria classified 78% as neighbours, 22% as intermediates and 0% as non-neighbours. Applied to the subjective intermediates, the objective criteria classified 12% as neighbours, 76% as intermediates, and 12% as non-neighbours. Applied to the subjective non-neighbours, the objective criteria classified some as neighbours, increasing the size of the subjective neighbour group by 3%, and some as intermediates, increasing the size of the intermediate group by 73%. Hence, the 2 methods agreed on more than three quarters of the neighbour assignments. Over-all, the objective criteria assigned an average of 3·7 neighbours and

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Fig. 1. Tracings of 3T3 nuclei in 3 frames from Film 18. × 150. Nuclei in Frame I (A) were numbered from 1 to 37, and followed to Frames II (B) and III (C). Nuclei present in Frame II but not in Frame I were assigned numbers 38 to 73, and followed to Frame III. Nuclei present in Frame III but not in Frames I or II were assigned numbers 74 to 96. Nuclei with letter suffixes are mitotic daughter cells. The letter codes were used to distinguish the 2 daughters by their relative positions at telophase: A, above; B, below; L, left; R, right. The cells designated 6, 13, 15 and 60 were binucleate. Fig. 1 A, Frame I, beginning of interval I, 37 cells; Fig. 1 B, Frame II, 2·2 days after Frame I, 64 cells, end of interval I and beginning of interval II; Fig. 1 C, Frame III, 2·2 days after Frame II, 68 cells, end of interval II and Film 18.

6·2 intermediates. In short, the objective criteria classified considerably more pairs as intermediate, obtaining 12% of the new intermediates at the expense of the subjective neighbour category, and 88% at the expense of the subjective non-neighbour category. Hence, the objective criteria will assign fewer neighbour losses and gains than would our subjective judgment, and thus are less likely to overestimate the amount of relative movement.

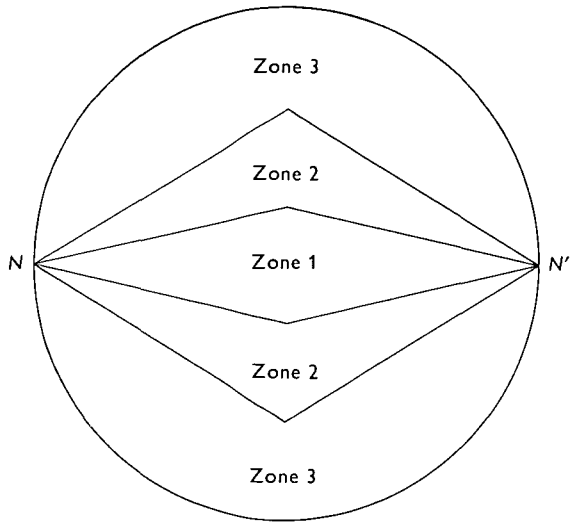

Fig. 2. Objective rules for determination of neighbour relationships. After considerable experimentation, the arbitrary diagram shown above was selected. (The height of the triangle formed by half of Zone 1 and the line joining points N and N' is $D/10$, where D is the diameter of the circle. The height of the triangle analogously formed by Zone 2 is $D/3$.) A series of drawings geometrically similar to the above figure were made for small increments in the diameter of the circle. These drawings were made on tracing paper, so that any pair of nuclei could be centred at points N and N' by selecting the diagram of appropriate size and laying it over the tracings of nuclei shown in Fig. 1. The positions of other nuclei were then used to determine the relationship assigned to the pair centred at N and N' by application of the following rules. Each nucleus is counted only once, in the innermost zone in which any part of it is included.

Neighbours: no nuclei in Zones 1, 2 or 3.

Intermediate: At least one nucleus in Zone 3 or Zone 2, but none in Zone 1. More than one in Zone 2 only if all are on the same side.

Non-neighbours: one or more nuclei in Zone 1, or 2 or more nuclei in Zone 2 provided both sides are occupied.

Selection of cells for neighbour analysis

The 37 cells in Frame I were numbered (Fig. 1A) and traced to Frame II. Among these 37 cells and their progeny, 20 mitoses occurred during this interval (one tripolar) adding 21 cells to the original 37, making 58 cells originating from those present in Frame I. Twenty-eight (48%) of these were still present in Frame II, 23 (40%) moved beyond the borders of the frame, and the remaining 7 (12%) died.

Some cells were sufficiently near the edge in Frame I or Frame II that not all their neighbour relationships could be assigned. When no cell occupied the equilateral

triangle defined by a cell and one or more edges of the frame, that cell was classified as an *edge cell* and was eliminated from the neighbour analysis. Using this objective criterion, 18 of the 28 cells traceable throughout the first interval were edge cells in Frame I or Frame II. This left 10 cells for neighbour analysis.

These 10 cells in Frame II were derived from 9 cells in Frame I. Four of the 10 cells in Frame II were daughters of divisions occurring during the first interval. Inclusion of the products of cell division in the neighbour analysis raises 2 questions: Do the dividing cells display an amount of movement different from the non-dividing cells? and Can division 'in-place', without relative movement, cause apparent neighbour exchanges, and therefore cause overestimation of the amount of relative movement? The answer to the first question concerns the behaviour of the cells, and will be discussed later. The answer to the second concerns the possible generation of false losses by methodological artifacts. Rules, as set out below, were adopted for scoring neighbour losses in cases involving cell division which prevent the overestimation of neighbour losses in this way, at the expense of sometimes underestimating them.

A. Division of the cell whose neighbours are being determined.
  1. If the daughters are neighbours,
    a. And both daughters are usable, consider the pair to be one cell, and reduce the gains, if any, by one.
    b. But one daughter is an edge cell, use the other daughter but reduce its losses, if any, by one; the edge daughter does not count as a gain.
  ii. If the daughters are intermediate, non-neighbours, or if one is missing, treat each separately.

B. Division of a neighbour.
  i. If both daughters are neighbours, reduce losses, if any, by one; the extra daughter does not count as a gain.
  ii. Loss of one daughter, but not both, does not constitute a loss.

C. Division of a non-neighbour: when both daughters are new neighbours, count only one as a gain.

In the case of the second interval, the initial frame (II) included 64 cells. Ten of these divided during the interval, making 74 cells originating from those in Frame II. Forty-five (61%) of these were identifiable in Frame III at the end of the second interval. Among the rest, 17 (23%) exited from the frame, 11 (15%) died, and the identity of 1 (1%) cell was lost by confusion with other cells. After eliminating all cells among the 45 present in both Frames II and III which were edge cells in either frame, 22 remained for neighbour analysis.

Neighbour losses and gains

Neighbour losses were defined as a change from 'neighbour' at the beginning of the interval to 'non-neighbour' at the end (see Fig. 2). Changes from 'neighbour' to 'intermediate' were not counted as losses. Neighbours which died during the interval were not counted as losses, since no relative movement is required for loss of the neighbour by death. Corrections for cell divisions were described above. Neighbour gains were

defined as a change from 'non-neighbour' to 'neighbour'. Changes from 'intermediate' to 'neighbour' were not counted as gains.

The average numbers of neighbours and intermediates per cell are summarized in Table 1 for both intervals. The average number of neighbours per cell was higher in Frame I than in Frames II or III. However, a neighbour must move out of the 'neighbour plus intermediate' category in order to be counted as lost. Since the 'neighbour plus intermediate' category is approximately the same size in Frames I and II, the reduction in average neighbours per cell during the first interval cannot be reflected as a net loss of neighbours. This is verified by the fact that gains (46%) equalled losses (48%) in this interval.

Table 1. *Neighbour losses and gains*

	Frame I	Frame II	Frame II	Frame III
Total cells present	37	64	64	68
Cells used in neighbour analysis	9	10	20	22
Total neighbours	33	26	53	61
Neighbours/cell	3·7	2·6	2·6	2·8
Intermediates/cell	6·2	8·0	6·8	7·5
(Neighbours plus intermediates)/cell	9·9	10·6	9·4	10·3
	Interval I		Interval II	
Duration, days	2·2		2·2	
Mitotic rate (mitoses/cell/day)	0·26		0·10	
Neighbours lost	16 (48 %)		20 (38 %)	
Neighbours gained	12 (46 %)		21 (34 %)	

Note. It is conceivable that, in occasional cases, a neighbour which is an edge cell might jostle out of the frame and back in again, without significant sliding relative to its neighbours. Its identity would be lost while out of the frame, however, and a new number would be assigned to it when it re-entered. Therefore it would be scored as a loss. This cannot have happened if the original neighbour is still identified in the final frame. Hence, the maximum possible extent to which exit followed by re-entry could contribute to the apparent losses can be subtracted by not counting losses for which the original neighbour is not identified in the final frame. This correction, which must produce a gross underestimate of exchange rate, reduces the losses in the first and second intervals to 24 and 28 %, respectively. The fact that the losses are still substantial, even after this over-correction, indicates that the majority of the losses observed were not artificial due to the exit and re-entry identification problem. Therefore, the best estimates of losses are those shown in the Table above.

In the case of gains, the numbers in the Table do not exclude gains by cells which lost an original neighbour by death. This is because presumably most of the cells replacing neighbours which have died come from the intermediate category, and therefore do not count as gains. This presumption is supported by the approximate equality of gains and losses in Table 1. (Even if 1 gain is subtracted for every neighbour death, the gains remain substantial: 35 % in the first interval, and 23 % in the second.) Therefore, the best estimates of gains are those shown in the Table above.

Neighbour losses and gains are shown in Table 1 and discussed further in its legend. Losses did not differ significantly from gains in either the first ($\chi^2 = 0{\cdot}03$, $P = 0{\cdot}85$) or the second interval ($\chi^2 = 0{\cdot}13$, $P = 0{\cdot}71$).

The over-all estimates are, then, that the average cell exchanged 47% of its neighbours in the first interval, and 36% in the second. The differences between the first and second intervals were not significant either for losses ($\chi^2 = 0{\cdot}96$, $P = 0{\cdot}33$) or gains ($\chi^2 = 1{\cdot}06$, $P = 0{\cdot}30$). Most cells lost some of their neighbours, while some cells lost all or none of their neighbours. The distribution of losses and gains is shown in Fig. 3.

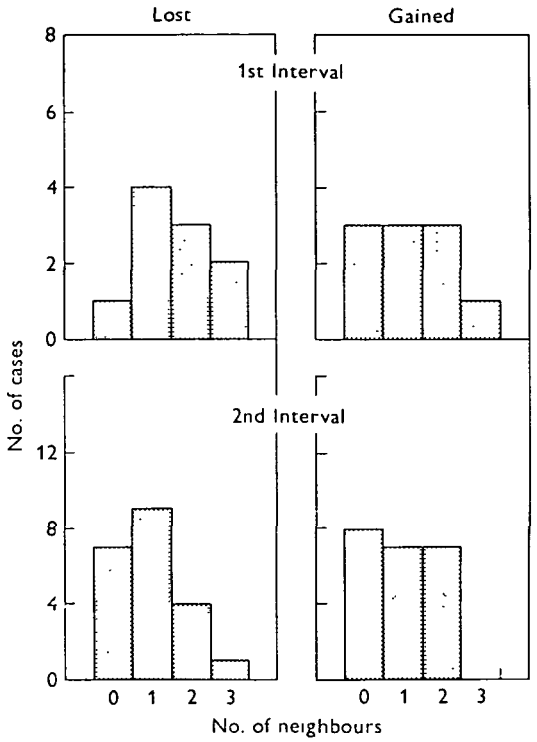

Fig. 3. Distribution of losses and gains. For definitions and explanation, see text.

Nuclear displacements

In a previous report (Martz, 1973) the speeds of nuclear translocation in Film 18 were measured (based on continuous tracings of nuclear positions) as a function of amounts of intercellular contact. Cells which were in contact with other cells on all sides had a mean nuclear speed of $8{\cdot}5 \pm 7{\cdot}7$ (S.D.) μm h^{-1}. Although persistent movement in a given direction was often visible in the film, jostling-in-place of the nuclei, without any net translocation, could also contribute to this mean speed. In order to find out whether net movement of the average nucleus was significant, the displacement of each nucleus was measured as the distance between its initial and final positions. Such measurements were possible for 28 cells in the first interval, and 44 in the second. The angular direction of displacement was also noted, and the mean vector displacement was computed for each interval. These means were 15 μm for the first interval and 28 μm for the second.

To compare these distances with cell size, it is useful to compute the mean cell

diameter. Since the cells are confluent, the substratum area occupied by the average cell can be closely approximated by dividing the area of the microscopic field by the average number of nuclei in the field during each interval. The square roots of these mean cell areas were taken as the mean cell diameters; they were 74 and 65 μm for intervals I and II, respectively.

Hence, we may state that the mean vector displacements were less than 0·5 mean cell diameters (the square root of the mean area per cell) in each interval. These means represent the net translocation of the entire population of nuclei, i.e. any slippage of the cell sheet as a unit, including any shifts in the position of the culture coverslip which might have occurred during filming. Since they are small, little such slippage occurred. Individual vector displacements were corrected for this slippage by subtracting the mean vector displacement from each. The resultant scalar displacements (irrespective of direction) were averaged to provide the best estimate of randomly directed nuclear displacement. The results were $1{\cdot}5 \pm 1{\cdot}0$ and $1{\cdot}1 \pm 0{\cdot}65$ mean cell diameters (corresponding to 2·1 and 1·4 μm h^{-1}) in intervals I and II respectively. These were both significantly different from zero (interval I: $t = 7{\cdot}9$, $P < 0{\cdot}0001$, $df = 27$; interval II: $t = 11{\cdot}2$, $P < 0{\cdot}0001$, $df = 43$). The mean corrected scalar displacement was significantly less in the second interval than in the first ($t = 3{\cdot}36$, $P = 0{\cdot}0013$, $df = 70$). The possible explanations for slowing down of cells during the film have been discussed previously (Martz, 1973). There was no significant correlation between the displacement of individual nuclei in interval I with their displacement in interval II, either in distance or direction. There were no significant differences between the mean displacements of nuclei in dividing and non-dividing cells in either interval or in the pooled data. In conclusion, each nucleus was, on the average, displaced more than one mean cell diameter in a random direction during each 2·2-day interval, which suggests that net cell movement was occurring.

Distances of separation associated with neighbour losses

We have shown that when the loss of a neighbour relationship is defined as the intervention of a third nucleus between 2 previously adjacent nuclei, a large percentage of neighbour relationships were lost during each 2·2-day interval. It may seem possible that these changes in the arrangement of the nuclei could have resulted from movements of nuclei within each cell's cytoplasm, while the cytoplasms remained anchored in place, and thus that no actual cell movement took place. If so, the nucleus which intervenes between 2 others (making the latter become non-neighbours) is simply moving into a region of its own cytoplasm which already intervened, and the 2 nuclei being separated need not necessarily move apart. In fact, however, the mean separation of pairs of nuclei which change from neighbours to non-neighbours increased from about 1 mean cell length to 3·3 in the first interval, and to 2·4 in the second interval (Fig. 4). The mean separation in the second interval was significantly less than in the first ($t = 3{\cdot}9$, $P = 0{\cdot}0007$, $df = 23$).

The increase in separation associated with neighbour losses was significant in both interval I ($t = 7{\cdot}0$, $P < 0{\cdot}0001$, $df = 14$) and interval II ($t = 5{\cdot}3$, $P < 0{\cdot}0001$, $df = 32$). This behaviour seems more consistent with net cell movement including

shifts of the bulk of the cytoplasm than with nuclear movements in a stationary cytoplasm.

More evidence against the stationary cytoplasm hypothesis is based on the number of nuclei intervening between 2 former neighbours. If intervention is by movement of the third nucleus within its own fixed cytoplasm, most neighbour losses should result from intervention of a *single* nucleus. In fact, however, about 70% of neighbour losses in the first interval and 40% in the second involved the intervention of 2 or more nuclei (see Fig. 5). The mean number of intervening nuclei associated with neighbour losses was $3{\cdot}1 \pm 1{\cdot}9$ in the first interval and $1{\cdot}9 \pm 1{\cdot}2$ in the second. The difference

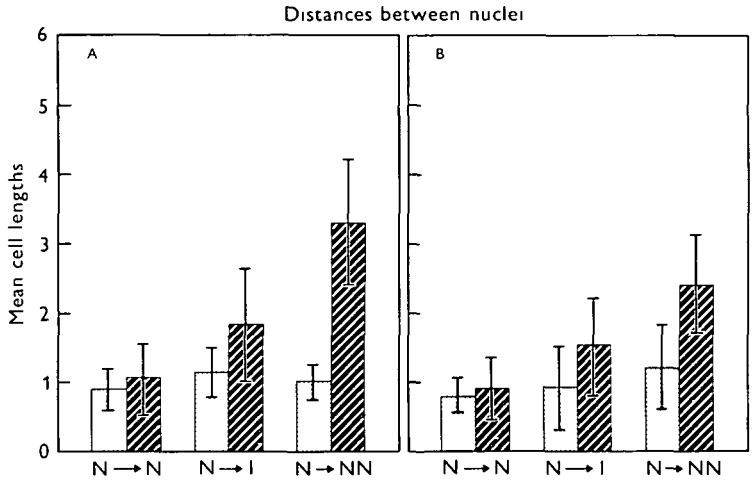

Fig. 4. Distances between nuclei before and after neighbour losses. Mean internuclear distances ($\pm$ S.D.) are shown for pairs of nuclei which were neighbours (N) at the beginning of each interval and which remained neighbours (N), or became intermediate (I) or non-neighbours (NN) by the end of each interval. Distances are expressed as mean cell lengths, which were 74 and 65 μm for intervals I and II (A and B), respectively. Mean values represent 8–17 measurements. Dotted areas, beginning of interval; striated areas, the end.

between the intervals was of borderline significance ($t = 2{\cdot}3$, $P = 0{\cdot}029$, $df = 30$). Five pairs in the first interval involved the intervention of 5 or more nuclei; 4 pairs in the second interval involved the intervention of 3 or more nuclei. In short, the behaviour of the nuclei strongly suggests that considerable net movements of whole cells took place.

Frequency of visible gaps between cells

Although the cells in the microscopic field were apparently 'confluent' throughout both intervals, small, brief gaps were occasionally observed. In order to quantitate these, 22 cells, selected because they were continuously observable for long time periods, were scrutinized for an average of 65 h each during the confluent phase of the film.

On average, each of these cells experienced 1·14 gaps associated with the rounding-up of an adjacent cell in mitosis. These gaps appeared to be clear of cytoplasmic

extensions, and their average duration was 35 min (equivalent to 0·9% of each cell's observation time). In addition, each cell experienced, on the average, 0·95 gaps associated with the death of an adjacent cell, lasting an average of only 8 min (0·2% of each cell's observation time). Occurrences were also seen which may have been gaps, and which were not adjacent to mitotic or dying cells, although it was difficult to

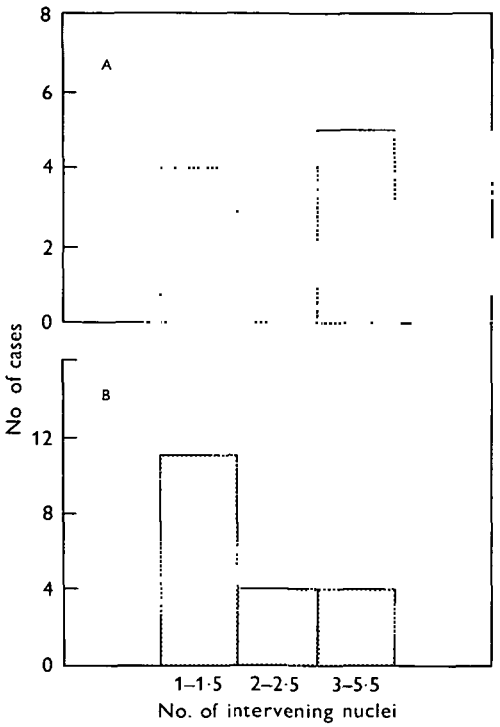

Fig. 5. Distributions of numbers of intervening nuclei associated with neighbour losses. Data were obtained for pairs of nuclei which changed from neighbours to non-neighbours. A, first interval; B, the second. The number of intervening nuclei was scored as the number of nuclei in Zone 1 plus one-half the number in Zone 2. Each nucleus was counted only in the innermost zone in which it was wholly or partially included. See Fig. 2 for definitions of these zones.

discern whether these occurrences were actual gaps free of thin cytoplasmic extensions. These occurrences appeared, on average, 1·14 times/cell, and lasted 60 min (1·7% of the cell's time). Occurrences in all 3 categories usually involved 10–30% of the cell's perimeter, occasionally up to 50%, and were seen in both intervals. In summary, including apparent gaps or possible gaps in all 3 categories, the average cell probably experienced one such occurrence approximately every 20 h and spent 3% or less of its time involved in such occurrences.

DISCUSSION

In previous reports, it has been demonstrated that 3T3 cells, under the essentially standard culture conditions used during Film 18 (Martz & Steinberg, 1972), display contact inhibition of speed of nuclear translocation (Martz, 1973), contact inhibition of nuclear overlapping (Martz, 1973), and post-confluence inhibition of cell division (Martz & Steinberg, 1972). In the present report, changes in nuclear positions have been analysed between the beginning and end of 2 intervals, each of 2·2 days duration. Although the cells were 'confluent' (see below) throughout both intervals, a mitotic rate of 0·26 mitoses per cell per day during the first interval was associated with an increase from 37 to 64 cells, while a reduced mitotic rate of 0·10 during the second interval maintained an essentially stationary cell number (64–68 cells in the field; for further discussion of growth properties of 3T3, see Martz & Steinberg, 1972). During each interval, the average nucleus was displaced in a random direction more than one mean cell diameter (taken as the square root of the mean area per cell). A substantial portion of the nuclei which were adjacent at the beginning of each interval became separated by other nuclei by the end of the interval. The number of nuclei which intervened between former neighbours was frequently as high as 3 to 5. Loss of original neighbour relationships in this way was associated with a 2- to 3-fold increase in the mean distance separating the originally neighbouring nuclei, and accompanied by the gain of new neighbour relationships between formerly distant pairs of nuclei.

All the measurements reported here are underestimates of the actual amounts of movement, both because of the stringent criteria established for neighbour gains and losses, and because our observations were necessarily restricted to nuclei which remained continuously in the field of view. Between a third and a half of the nuclei moved beyond the limits of the field during each interval, and these probably included many of the fastest moving nuclei. These observations strongly suggest that considerable net movements of whole cells were occurring in random directions, despite the confluence and contact inhibition of nuclear overlapping. (A seemingly unlikely alternative interpretation would be that most cell cytoplasms are spread over much more than one mean cell length, resulting in extensive overlapping of thin cytoplasmic sheets. Then it would be conceivable that each nucleus could have made the observed movements within its own cytoplasm, while the cytoplasmic borders of each cell remained anchored in place.)

Two general categories of explanation have been offered to explain contact inhibition of overlapping. One states that the cell's locomotory apparatus is locally paralysed in whatever regions of its periphery are contacted by other cells (Abercrombie & Heaysman, 1954; Abercrombie & Ambrose, 1958; Abercrombie, 1961, 1967, 1970). That such a paralysis does not encompass free regions of the cell periphery is clear from the fact that cells are usually observed eventually to pull away from an intercellular contact, and to continue their movement in a direction not obstructed by contact with other cells, when such a free edge exists (Loeb, 1921; Abercrombie & Heaysman, 1954; Abercrombie & Ambrose, 1958; Trinkaus *et al.* 1971). Observations with light and electron microscopy have revealed a sequence of rapid changes in the

region of a cell's cytoplasm making contact with another cell (Abercrombie & Ambrose, 1958; Abercrombie, 1970; Heaysman & Pegrum, 1973). However, it is by no means self-evident that these events are essential for or contribute to the failure of overlapping, persisting over many hours or days, which is the subject of our present attention.

The second category of explanation states that the inhibition of overlapping results from differential adhesion. If the intensity of cell–substratum adherence exceeds that of cell–cell adherence, cell movement may be secondarily restrained by the adhesive energy differential which the cell's locomotory apparatus would have to overcome in order to produce overlapping with another cell (Abercrombie & Heaysman, 1954; Abercrombie, 1961; Carter, 1965; Garrod & Steinberg, 1973; Martz & Steinberg, 1973). We have recently detailed a physically explicit version of this hypothesis (Martz, Phillips & Steinberg, 1974). These alternative explanations for contact inhibition of overlapping are contrasted by the question: Does the avoidance of overlapping result from local inhibition of locomotion (the local motor paralysis explanation), or does inhibition of locomotion across an obstructing cell result from avoidance of overlapping (as would occur by the differential adhesion explanation)?

The observation that cell locomotion apparently continues in confluent populations of cells which strongly avoid overlapping, such as 3T3 studied here, and embryonic chick liver cells studied by Garrod & Steinberg (in preparation), shows that extensive intercellular contact around a cell's perimeter very likely does not prevent its locomotion. This is somewhat difficult to reconcile with the local contact paralysis hypothesis, in which immobilization would be expected if a cell is continuously in contact with other cells around its entire periphery. However, what appeared to be small, brief gaps between cells were occasionally observed. Quantitative scrutiny revealed that on the average each cell experienced such an occurrence at 20-h or longer intervals and spent not more than 3% of its time involved in such occurrences. Smaller gaps, not visible in our film, may also have occurred. Whether or not such gaps are important to the nuclear movements observed cannot be answered at this time, but it should be noted that such gaps may be the rule rather than the exception among 'confluent' monolayers. The term 'confluent' as used in the present context has not been previously defined either to exclude the existence of such small gaps, or at the ultrastructural level.

Nevertheless, it seems to us implausible, in view of the low incidence of observed gaps, that extensive cell movements would be permitted within the monolayer studied here if regional paralysis of a cell's locomotory machinery, sufficient to prevent overlapping over long periods of time, occurs wherever intercellular contact is present on the cell periphery.

In contrast, the cell movements which apparently occurred in these confluent monolayers would be expected if avoidance of overlapping (due to differential adhesion) is the primary event, rather than an inhibitory influence of cell contact on the cell's locomotory apparatus: the cell would be free to move in any direction by sliding between the surrounding cells so long as it does not overlap very far with any of them (move off substratum and on to another cell). Both the avoidance of over-

lapping and the mechanical resistance to moving among adherent neighbours would be expected to reduce cell speed, as has been observed here and elsewhere (Abercrombie & Heaysman, 1954; Martz, 1973), but not to prevent movement altogether.

In conclusion, it has been shown that extensive nuclear movements, strongly suggestive of cell movements, occurred in a confluent monolayer of 3T3 cells. Although the evidence presented here does not exclude the local contact paralysis hypothesis for contact inhibition of overlapping, differential adhesion seems a more plausible explanation of the behaviour observed. These results bring into focus the importance of evaluating whether or not any correlation exists between the nuclear movements of individual cells and the occasional gaps between cells which apparently occur in 'confluent' monolayers.

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