THE ANGULAR DISTRIBUTION OF DIRECTIONAL CHANGES

OF GUIDED 3T3 CELLS

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ABSTRACT

This paper reports that the angular distribution of directional change of 3T3 cells which followed guiding lines on a substrate and left the guidance at various angles shows a peculiar preference for angles between 30° and 60°. Regarding the phenomenon of cellular guidance itself, 3T3 cells faced with a choice between guiding lines toward different directions seem to explore various options before following one. This observation suggests that the outcome of a probing action and not substrate properties alone can explain the guidance behavior of 3T3 cells.

KEY WORDS   3T3 cells ·   contact guidance ·
phagokinetics ·   angles of directional change

Animal cells respond to anisotropies on the substrate by orienting or even migrating along them. Various explanations for this phenomenon, called "contact guidance" (13), have been suggested in the past; and its importance for malignant invasion, immune response, and embryonic development has been emphasized (6, 14). Explanations include guiding influences of microexudates on the cell surface (14), a local weakening of the cell surface which contacts a borderline between two substrate areas of different quality (13), influence of oriented submicroscopic lines on the substrate which direct a flow of metabolic energy along cell surfaces (14), differential adhesion of cells around their perimeter (6), topological factors (7), and the inability of microfilament bundles to follow excessive substrate curvature (8).

As yet, there is no agreement among investigators of the phenomenon regarding which explanation is most fundamental. However, several investigators have emphasized that adhesive properties of the substrate alone can hardly explain the guidance behavior of cells (10, 14), particularly not in the special case of a substrate anisotropy in the form of another cell which comes in the way of a migrating cell (1).

My present report about the guidance behavior of 3T3 cells on a specially designed substrate does not intend to suggest a further mechanism of contact guidance. Instead, I wish to propose that there is a cellular "steering" system in 3T3 cells which produces directional changes with a peculiar angular preference, regardless of whether the cells are guided or not. Furthermore, if 3T3 cells are faced with a choice between two guiding lines which are identical except that they guide the cells in different directions, the cells seem to "explore" various options before following one.

The guiding substrate used was designed to be reproducible, to have a large experimental variability, and to be compatible with high-resolution light microscopy and the previously described technique of phagokinetic tracks (3, 4). I found such a substrate in glass coverslips which were covered with a 290 Å-thick, homogeneous layer of evaporated gold. With a fine diamond tip, thin lines were scratched into the gold layer which exposed the underlying glass surface without, however, scratching it. Thus, the depth of each scratch was reproducibly 290 Å. The direction of the lines and their mutual distance could be reproducibly varied. The guidance behavior of cells on such substrates was recorded by their phagokinetic tracks. Therefore, as required by the technique, an additional thin layer of denatured bovine serum

albumin, followed by a layer of supra-colloidal gold particles, was placed on top of the scratched guiding substrate.

MATERIALS AND METHODS

Substrates for phagokinetic tracks were produced as previously described (3, 4). Plain glass substrates were precoated with a 1% BSA solution (bovine serum albumin crystallized, Schwarz/Mann Div., Becton, Dickinson, & Co., Orangeburg, N. Y.) before adding the particle suspension in the described way, whereas glass substrates covered with layers of evaporated gold (see below) were precoated with a 2% BSA solution.

To produce substrates for contact guidance, 22 × 22-mm² coverslips (Coverglass No. 1½, Corning Glass Works, Science Products Div., Corning, New York) were placed on a rotating stage in a Denton DV-502 evaporator (Denton Vacuum Inc., Cherry Hill, N. J.) and 56 mg of 8-mil gold wire (Ladd Research Industries, Inc., Burlington, Vt.) were evaporated 18 cm away from the center of the stage, which was rotating at a speed of 30 rpm. The estimated thickness of the evaporated gold layer was 290 Å ± 30 Å. Subsequently, the gold-coated coverslips were placed on a mechanical device to produce sets of parallel scratches in the gold coat. This device was built in collaboration with Mr. B. DeTroy, Cold Spring Harbor Laboratory. Details will be furnished on request. Briefly, it consisted of a microscope stage which was pneumatically shifted back and forth in the x-direction. At the end points of each sweep, a stepping motor (The Superior Electric Company, Bristol, Conn.; power supply MPS 1000, indexer PIM 151, stepping motor M061-FC08) advanced the micrometer in the y-direction by a preset number of steps. The coverslip was held on a small vacuum stage which could be rotated to adjust various angles with respect to the direction of the x-sweeps. A diamond stylus (phono point diamond, heavy duty, 60°, ⅛ SH., Serial 3539, Manhattan Supply Company, Plainview, New York) in a fixed position was pressed on the coverslip under its own weight of 15.6 g, thus scratching the gold coat of the coverslip, which moved under it, without scratching the glass carrier. The width of the scratches was 17 ± 4 µm; the distance between scratches was adjustable in steps of 30 µm. There was some inevitable wobble in the stylus which caused variations of ± 20 µm. Therefore, the minimal settings used in the experiments were two-step settings. After scratching the coat of evaporated gold in the desired way, the coverslip was sintered for a few seconds over an ethanol flame until the color of the gold layer changed from green to blue-grey. Subsequently, the coverslip was coated with gold particles in the previously described manner. 3T3 cells, a kind gift of Dr. Howard Green, Massachusetts Institute of Technology, were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum (Grand Island Biological Co., Grand Island, N. Y.) in a 10%

CO_2 atmosphere and were subcultured every 3 days in 6-cm Falcon plastic dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) by suspending the cells in a 0.05% solution of trypsin and 0.5 mM ethylene-dinitrilotetraacetic acid (EDTA) in phosphate-buffered saline (PBS). Experimental cells were plated into 3.5-cm Falcon plastic dishes which contained the test substrate and 2 ml of medium. The passage number of the experimental cells was between 8 and 22.

RESULTS

Fig. 1 a and a' show a typical track produced by a 3T3 cell on a guiding substrate as described above. It is obvious that the cells follow the guiding scratches for some distance but are able to leave them by turning at certain angles. Similar behavior could be observed on platinum layers (Fig. 1 b and b'). Observations of live cells (Fig. 1 c–e, c'–e') showed that cells which followed scratches generally extended to both sides into the gold area while keeping the nucleus approximately centered over the scratch. Some cells became rather spindle-like; others assumed a more rounded shape than they would on normal substrates. Their method of locomotion involved the usual formation of a leading edge and a retracting tail as on normal substrates (2).

The Guidance of 3T3 Cells along Straight Lines

If one was to explain this cellular behavior entirely by a passive cellular reaction (attraction or retardation) to the areas of evaporated gold, one would expect that a cell passing a borderline between glass and gold should change direction in a consistent way. Once a cell passing the borderline between two substrates is located with half its body on one substrate and with the other half on the other substrate, its speed of locomotion should decrease in the half which contacts the retarding substrate. As a result, the cell body would turn and orient itself more perpendicular to the borderline. The identical explanation holds for, e.g., a light wave passing into a medium of higher refractive index. The mathematical formulation of the behavior of any extended object passing the borderline between two media of different speeds of propagation is in each case similar to the law of refraction. Consequently, I examined whether any attraction or retardation of the cells on gold substrates would be strong enough to create guidance by testing whether there was a "refractive index for cell migration." In this test, I used half-

evaporated substrates, as shown in Fig. 1f, f', g, and g'. If an attracting or retarding influence of one type of substrate as compared to the other would be the dominating cause for directional changes, a plot of the sine of the angle on the gold (sin W_{go}) versus the sine of the angle on glass (sin W_{gl}) (see Fig. 1f-g') should show a correlation similar to the law of refraction. Evaluation of these data from 74 tracks similar to the ones on Fig. 1f-g' showed no linear correlation. (A nonlinear correlation may have been obscured by the scatter of data points.) This is not to say that there is no attracting or retarding influence of the different substrates on 3T3 cells but that directional changes cannot be explained solely by them.

Nor did the additional presence of the gold particles on the substrates seem to contribute to the observed guidance as tested by counting the percentage of cells inside scratches on substrates with and without additional gold particles. In the test substrates, the ratio between the area of plain glass as exposed by the scratches and the area still covered with evaporated gold was 1:3.4. A count of 240 cells on a scratched substrate with or without additional particles yielded in both cases a ratio of approximately 1:1, which indicates that the presence of the particles did not influence the cells' apparent threefold preference to locate themselves above the scratches.

The Turning of Guided Cells

The cells follow the guiding lines for various distances; the longest distance found was 1,000 μm. Measurement of 213 values yielded an asymmetrical distribution of straight-track segments along guiding lines with a mean of 280 μm and a standard deviation of 150 μm.

One may think of inhomogeneities along a guiding scratch as the cause for the cells' turning out. Such inhomogeneities should be approximately a cell's size because smaller inhomogeneities, like the particles themselves, do not disturb the guided migration. However, as shown in Fig. 1e, no such sized inhomogeneity is visible. Another explanation for the turning of cells out of guiding lines may be sought in variations of the height (290 Å) of a scratch's edge. Substantial height differences could not be observed in substrates as judged by alterations of light extinction in the gold layer. Furthermore, the evaporation of a second thin layer of gold on top of an already scratched substrate should not affect the guidance

behavior of the cells if the groove profile was responsible for the guidance, because the second evaporation would preserve the profile. In contrast, I found that cells on such double-evaporated substrates showed no guidance behavior. This observation suggests that the cells did not respond to the profiles on the substrate but rather to unspecified influences exerted by the borderlines between the gold and the glass surfaces.

Another explanation may be that a cell follows a scratch as long as its extensions to both sides into the gold areas remain equally large. If, by accident, one becomes larger than the other, the cell may be retarded or accelerated on one side and in this way become "derailed." However, many cells followed a single borderline between gold and glass while contacting gold on only one side (Fig. 1g), yet according to the above explanation for a cell's turning, these cells should derail continuously.

Measuring the angles w_1 at which cells moved into or out of guiding lines yielded essentially identical angular distributions. Therefore, both distributions were combined into one which is shown in Fig. 4a. This angular distribution of directional change shows a peak between 40° and 60° and decreases gradually towards 180°.

Turning of Cells into Free Space

The above method of measuring directional changes may be considered biased because it seems possible that directional changes of angles smaller than 20° remove the cells too slowly from the guiding lines and thus they are guided back to it soon after.

To measure especially small angles of directional changes, one may use a substrate which allows the cell to move into a guidance-free space after following a guiding line. Examples of such substrates with phagokinetic tracks are shown in Fig. 2. 199 tracks of cells which had followed a guiding line until they reached the free space showed that 21% did not turn before having migrated some distance straight ahead into the free space (Fig. 2a, left track), whereas 34% chose the borderline between gold and glass as a new guiding line to turn at right angles and follow for some time. Therefore, the angular distribution for this type of directional changes shows two sharp peaks at 0° and 90° (Fig. 4b). In between, however, one can see again a preference of angles w_2 between 30° and 60°. Angles greater than 90°
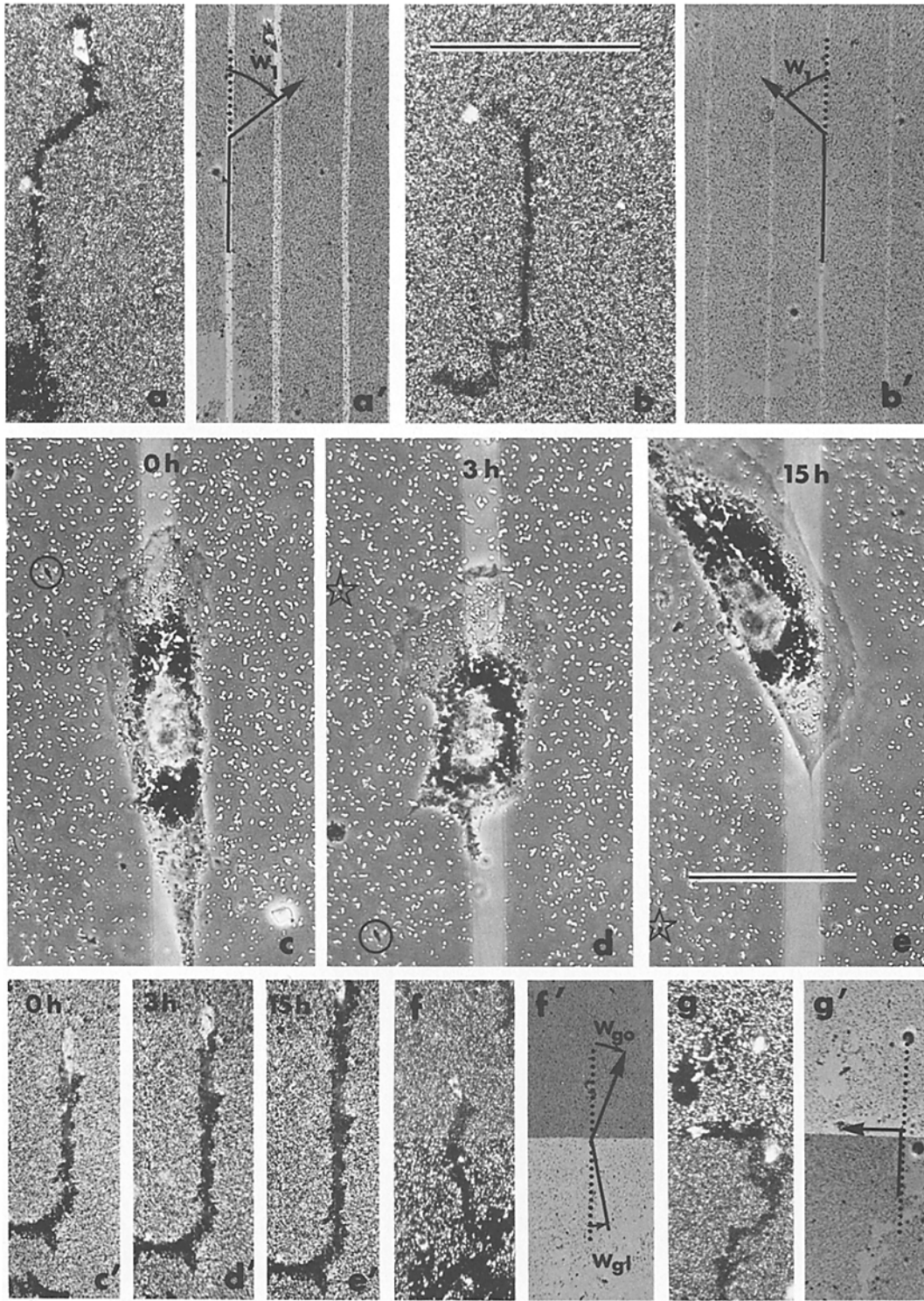
were not counted in this assay because cells turning backwards at the borderline could not be distinguished from cells turning before reaching it.

The Change from One Guiding Line to Another

In both types of substrates used in the above experiments, a cell loses its guidance after a directional change. One can also study directional changes in cases where the turning cells regain guidance with substrates as shown in Fig. 3. In this case, there were two sets of parallel scratches which crossed each other at a prefixed angle. A cell which followed a guiding line and approached the intersection between two lines had the options of continuing straight ahead, of turning at the prefixed angle w_3, of turning at the complementary angle $180°-w_3$, of migrating backwards, or of leaving the guiding lines by moving into one of the diamond-shaped gold areas. For each prefixed angle, the distance between intersections was adjusted to be about 280 μm, which was mentioned above as the average distance that the cells follow guiding lines before making directional changes. By counting only the cells which arrived at an intersection after having followed a guiding line for some distance, I found that very few cells migrated backwards or left the guiding lines at intersections; often they left the lines between intersections, and others produced long tracks within the network of lines as shown in Fig. 3a–c. To test quantitatively the behavior of cells which arrived at intersections, I used in each experiment different substrates with different preset angles w_3 of intersecting lines and counted the number of cells which passed straight through the intersec-
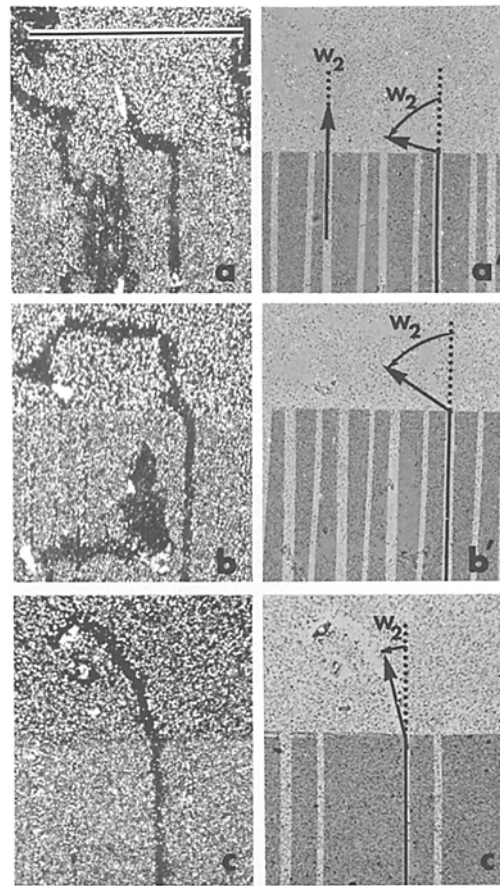

FIGURE 2 Phagokinetic tracks of 3T3 cells moving along guiding lines into free space, performed to measure especially the small angles w_2 of directional change. The right-hand panels show the same frames as the left-hand panels in brightfield light microscopy (Bar, 500 μm).

FIGURE 1 Phagokinetic tracks of 3T3 cells guided along scratches in a layer of evaporated gold on a glass coverslip. The darkfield light micrographs (a, b, f, and g) are accompanied by brightfield micrographs of the same frame. (a, a') Track of a guided 3T3 cell which leaves the guiding line at a certain angle w_1 as illustrated in the brightfield image of the same area (panel a'). (b, b') Similar behavior of 3T3 cells on scratches in a layer of evaporated platinum (Bar, 500 μm). (c–e) Live cell observation of a 3T3 cell migrating along a scratch in an evaporated gold layer. Circles and stars mark points on the substrate to demonstrate the advancing of the cell. Lamellipodia extend to both sides of the scratch while the nucleus remains approximately centered over it. The cell migrates with a normal leading edge and a retracting tail (cf. c, d). After 15 h, the cell turns off the scratch at an angle of ~30° without any visible inhomogeneity on the substrate as possible cause (Bar, 50 μm). (c'–e') Elongation with time of the phagokinetic track of the same cell as shown in panels c–e. (f–g') Change of direction of phagokinetic tracks of 3T3 cells passing the borderline between a glass substrate and an area of evaporated gold, performed to test whether any of the two substrates has a consistently retarding effect on cell migration relative to the other.
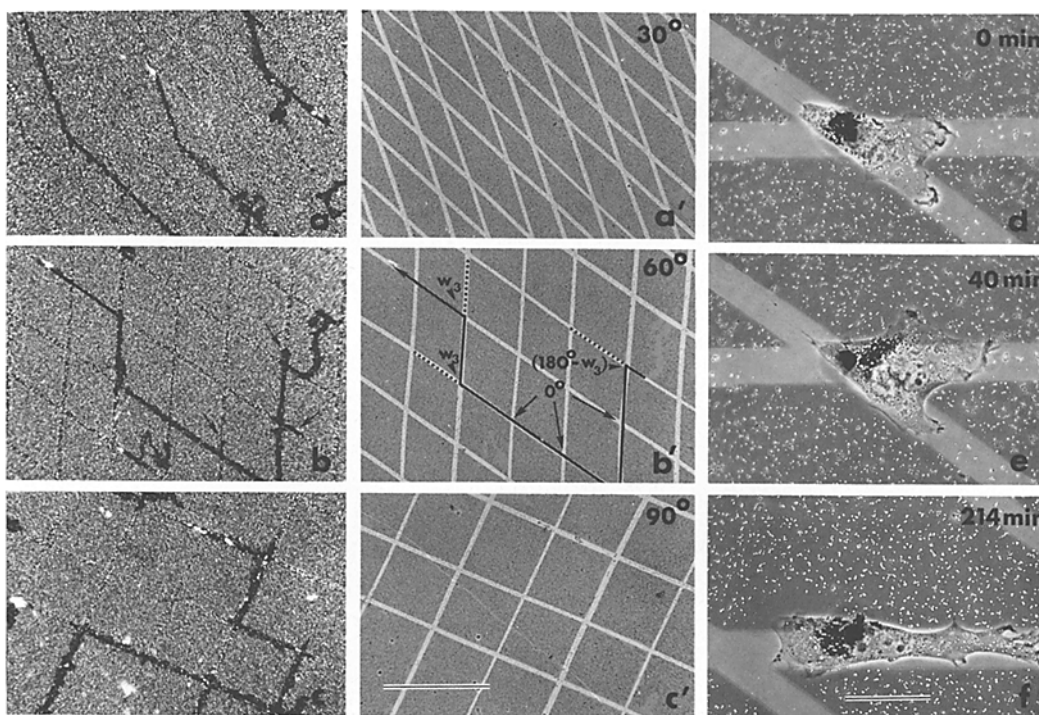
FIGURE 3 (a-c') Phagokinetic tracks of cells which regain guidance after directional changes if they migrate along the network of lines as shown in the middle panels. Various different angles w_3 of crossing between guiding lines were offered to the cells, and their tendency to follow the preformed angle was quantitated by counting the number of cases in which they turned at this angle w_3, or its complement $180°$-w_3, or in which they continued straight through an intersection of guiding lines (Bar, 500 μm). The depicted tracks were produced during 51 h of incubation. Note that the beginning of the track in panel b leads across an area of evaporated gold. (d-e) Observations of a living 3T3 cell at the intersection between two guiding lines crossing at 40°. (Bar 50 μm) The cell is coming from the upper left corner. After removing the gold from the guiding line to its right hand side, it extends lamellipodia into the two other optional directions (d). Subsequently, it retracts the lamellipodium which points into its foreward direction and elongates along the guiding line at 40° to its left. This "exploration" of optional directions is expressed in the phagokinetic tracks as little thorns pointing to the side of a track at intersections (see arrows in panel b).

tion, or turned at the preset angle w_3 or its complement $180°$-w_3. The counting procedure used the phagokinetic tracks in which the cells had recorded their choice at each intersection they had passed along their way. In nine independent experiments, the percentage of cells which turned at the preset angle w_3 or at its complement $180°$-w_3 in a total of 2,208 encounters of cells with an intersection yielded the curve shown in Fig. 4c. (Standard deviations are ~0.08 for each point, yielding an error of the mean of 0.03.) The peak of the angular distribution is quite broad, presumably because the regained guiding influence tends to equalize the cells' preference to certain angles of directional change. However, one can still

discern a preference for angles between 30° and 60°.

Live cell observations showed that the quite intriguing action of cells at intersections extended into the various possible directions before following one of them (Fig. 3d-f). Such "exploring" activity can also be seen recorded in the phagokinetic tracks as short thorns extending into various preformed directions at intersections (see arrows in Fig. 3b).

Directional Changes in Freely Migrating 3T3 Cells

I also tested directional changes in freely migrating 3T3 cells along their phagokinetic tracks

in the presence of 10 or 20% calf serum. The difficulty in accurately measuring directional changes in free tracks lies in the precise definition of a straight-track segment. There are some ambiguities in the position and size of a directional change along a track with bending segments, which can cause considerable experimental errors. Assuming that the ambiguous cases would not overly distort the histogram of directional changes in free tracks, I measured 531 cases and obtained a curve quite similar to Fig. 4a (Fig. 4d).

DISCUSSION

Gail and Boone (9) have published earlier angular distributions of directional change of 3T3 cells migrating on plain glass surfaces. Their method of measuring the angle of directional change was to connect consecutive positions of cells at 2.5- and 5-h intervals, thus determining the chord angles of the actual pathway, although not the true angles

of directional change. Replotting the data in their Figs. 3 and 4 by absolute values of angles yields distributions which suggest a preference of angles between 20° and 60°. Nossal and Zigmond (11) found that polymorphonuclear leukocytes (PMN) show a marked preference for directional changes between 30° and 50° if migrating in a chemotactic gradient. According to Peterson, Hall, and Nobel (1978, personal communication), the same preference can be found in PMNs in the absence of such gradients.

If, therefore, guided and nonguided 3T3 cells as well as PMNs in the presence or absence of a chemotactic gradient likewise make directional changes with a similar angular characteristic, it seems possible that these are produced by a cellular steering mechanism which has this characteristic, regardless of strong environmental influences. Instead of a steering mechanism, one may also think of influences of the cytoskeleton
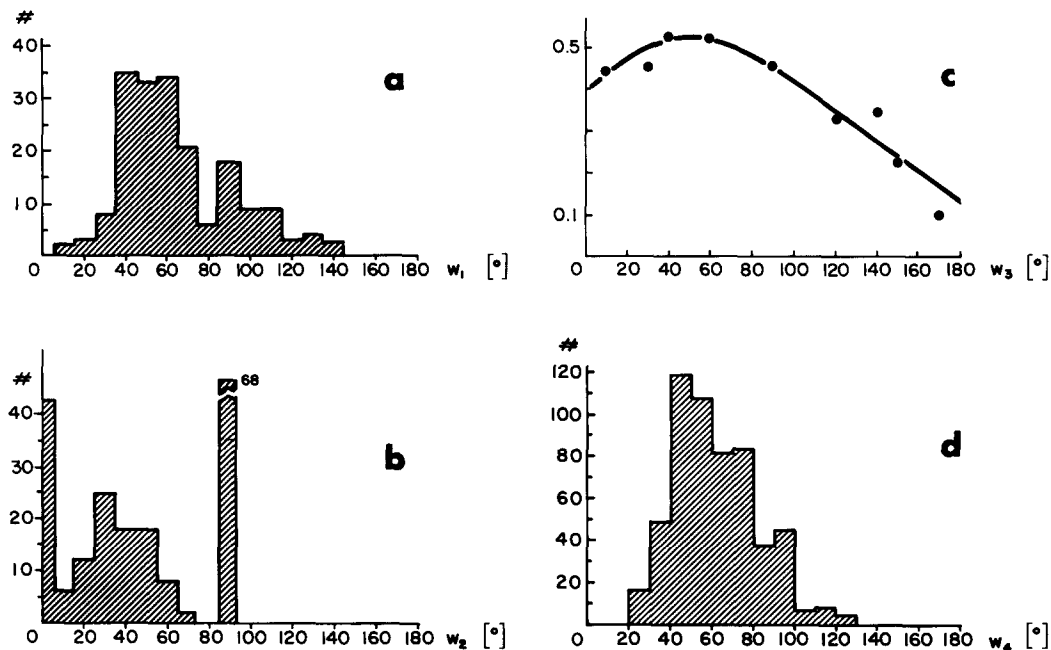

FIGURE 4   Angular distributions obtained from phagokinetic tracks on the three substrates depicted in Figs. 1, 2, and 3. (a) Combined histogram of the angles and number of cases observed at which cells turned into or off guiding lines (192 values). (b) Histogram of the angles at which cells turned from guiding lines into free space. The peak at 0° corresponds to cases where cells did not turn before moving straight ahead for various distances into the free space (e.g., Fig. 2a, left-hand track); the peak at 90° corresponds to the cases where the cells accepted the borderline between free glass and scratched gold as new guidance (131 values). (c) Fraction of cells which followed a preformed angle on scratched substrates as shown on Fig. 2 (a–c'). Error of the mean is ~0.03. The curve was obtained from 2,208 encounters of cells with an intersection between guiding lines. (d) Histogram of 531 directional changes of 3T3 cells measured along phagokinetic tracks in the absence of guidance.

on the angle of directional changes. It seems possible that the necessary rearrangements of the cytoskeleton during and after a turn favor angles of 30°-60° over others.

In previous presentations, I have published observations that major directional changes along the tracks of 3T3 cells appear predetermined in the cells (3, 4). Cell-cell collisions can override this predetermination (4). The present paper adds two more aspects to the control of migration in 3T3 cells.

The guidance behavior itself shows another example of overriding exogenous influence on the predetermination of migration, because the straight-track segments produced by guided cells would not have occurred without the lines on the substrate. Yet, the cells in turn appear able to override the guiding influence as shown by their turning off the guiding lines. Furthermore, if faced with alternatives of guiding lines, they seem to probe their various options before following one. Taken together, it seems that the following picture of migrational control in 3T3 cells emerges: Whether a cell turns, at what time, and to which side this happens depends on endogenous predeterminations, exogenous influences, and the outcome of cellular probing actions. The endogenous predetermination dominates only in the absence of any exogenous input, e.g., on a homogeneous substrate and away from other cells (4, 5). However, in case the cell initiates a turn, it seems to be carried out by a cellular system with an angular preference between 30° and 60°.

A control system of migration with such properties appears quite complex, indeed. Yet, considering the various morphogenetic movements of animal cells, this degree of complexity may not seem surprising. Which cellular organ could be a candidate for such a control system?

For the following reasons, one might consider the centrioles in this respect: (a) The particular area of the perimeter of a migrating fibroblast which becomes its leading edge is dependent on the intactness of the cytoplasmic microtubules (12). Many cytoplasmic microtubules in turn appear to be focused around the centrioles in animal cells. (b) The primary cilium which grows intracellularly out of one centriole of a pair points predominantly in the direction of migration in 3T3 cells (5). (c) The well-known ninefold rotational symmetry of centrioles divides their perimeter into equal sectors of $360°/9 = 40°$. This paper and other papers mentioned (9, 11) show an angular preference of directional change of $40° \pm 20°$. Further investigations are underway to define the possible role of centrioles in the control of cell migration.

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