FILOPODIA OF SPREADING 3T3 CELLS

Do They Have a Substrate-Exploring Function?

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ABSTRACT

Freshly plated 3T3 cells send out radial projections or filopodia. We observed cells which happened to settle on glass near the borderline of a gold-plated area. When some of the filopodia contacted the gold-plated area and others the glass substratum and remained attached for a few minutes, lamellipodia then extended preferentially toward the gold-plated area. 1-2 h later, most of the cells were found in the gold-plated area. When the filopodia of a spreading 3T3 cell contacted another already spread 3T3 cell and also the glass substratum, the first lamellipodia extended preferentially towards the glass. These observations suggest a directionally differentiated extension of lamellipodia after the filopodia of a spreading 3T3 cell have contacted different substrates in their environment. Before filopodia contact a substrate, they perform a rapid "scanning" motion. Therefore, we suggest that the filopodia of a spreading 3T3 cell serve as organs which explore the nonfluid environment and react to a certain quality of the substrate that is presently unknown. Subsequently, they mediate the extension of lamellipodia into the direction in which this quality is found. The described phenomena are reversibly inhibited by Cytochalasin B at concentrations above 5 μg/ml although filopodia are produced.

Single embryonic cells should be able to examine their vicinity, and there is growing evidence that they do (17, 29). Anchorage is a prerequisite for the proliferation of normal tissue culture cells (21, 25), most of which are derived from embryonic tissue, and the cells indeed move into areas of high adhesion (8). Normal fibroblasts avoid overlap with other cells (2, 29), and growing neurites seem to be able to avoid collision with another neurite (14).

In order to explain these phenomena, one may begin to search for an "exploratory cellular organ", i.e., a cellular organ which is capable of interacting with different sections of the substrate around a cell and of mediating a cellular reaction after it has "detected" a favorable area. Even though we do not presently know what (local) quality of a substrate should be considered favorable, a cellular structure may be considered a good candidate for an "exploratory organ" if it fulfills the following criteria: (a) it performs a rapid scanning motion before it attaches, and can attach to various substrates; and (b) a directionally differentiated cellular response can be observed although the cell is completely surrounded by the same kind of substrate, and only the scanning organ is in contact with another kind of substrate at some distance from the cell body.

There are already several suggestions in the literature that the fibrous extensions of 0.2 μm diameter and varying lengths between 2 and 30 μm which emerge from the cell surface are good candidates for an exploratory organ of single embryonic cells (5, 10, 11, 13, 14, 17, 20, 28).

These extensions were first described as "fibrous projections" by Porter, Claude, and Fullam in 1945 (22). Since then, the terminology has varied considerably. In the literature there are reports on "finger-like projections" (12), "microfibrils" (15), "microspikes" (31), "numerous and extremely thin pseudopods" (17), "microextensions" and "retraction fibrils" (26) and "filopodia" (27). We prefer to use the term "filopodia" because it matches the term "lamellipodia".

We studied the behavior of filopodia during spreading of 3T3 mouse fibroblasts on a solid, extended substratum. Freshly trypsinized cells, after suspension for less than 1 h, appear in the light microscope as small spheres surrounded by many limp fibrous structures. These structures appear to be "retraction fibres" which formed while the cells rounded up under the influence of trypsin (23) and were detached together with the cell body by the subsequent suspension process. 10 min after the suspended cells have settled on a new substratum, hardly any fibrous structures are visible. Another 10–20 min later, however, the cells have again sent out thin fibrous projections which we call filopodia. These filopodia appear rigid and perform a rapidly scanning motion until they attach to the substratum. Subsequently, the cells extend lamellipodia towards the areas where filopodia have attached. Cytoplasm from the spherical cell body flows into the extended lamellipodia, and eventually the cells assume again a flat, extended morphology. We suspect that the scanning motion of the filopodia expresses the cells's search for anchorage points near the spot where the cell has settled by accident.

Previously, we studied these events on a substratum which was densely and evenly covered with loosely attached colloidal gold particles before inoculation with cells (4). We found that the filopodia of freshly plated 3T3 mouse fibroblasts removed the gold particles in a circular area around each spreading cell before the cells extended any lamellipodia. In the presence of Cytochalasin B (CB) the filopodia were still present in the spreading cells, but the gold particles around the cells were not removed even though the particles appeared to stick to the filopodia (4). This finding may suggest that the force which removes the particles could not be produced by filopodia in the presence of CB.

At first sight, the removal of such particles may seem unrelated to an exploratory function of filopodia. Observation of living cells, however,

gives the impression that the contact between filopodia and particles mediates the production of a certain retraction force which is required for the centripetal transport of the particles. It seems possible that filopodia produce the same retraction force if they do not attach to loose pieces of solid gold but to an extended solid gold substratum as well. If the attachment between filopodium and substrate is firm enough, the retraction force will not produce a shortening of the filopodium. However, it may elicit a cellular response which shows directionality if only the filopodia and not the cell body are in contact with the gold substrate. Therefore, we prepared glass substrata with gold particles which were confined to certain areas and attached too firmly to be removed. Subsequently, we studied the extension of lamellipodia of freshly plated 3T3 cells which happened to settle entirely on glass and whose filopodia contacted both glass and gold. In another type of experiment, we observed the extension of lamellipodia of freshly plated 3T3 cells whose filopodia has contacted both glass and another cell. In order to further test the involvement of the postulated contact-mediated retraction force in the observed cellular behavior, both types of experiments were also carried out in the presence of CB.

MATERIALS AND METHODS

3T3/Swiss cells were a gift from Dr. R. Pollack, Cold Spring Harbor Laboratory. They were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Microbiological Associates, Inc., Bethesda, Md.) at 37°C in a 5% CO_2/air mixture. Mycoplasma contamination was permanently removed from the cells by a single incubation of stock cultures at 41°C for 24 h (Albrecht-Buehler, G., M. Osborn, and K. Weber. Unpublished observations). The cells reach saturation densities of 40,000/cm² (without medium change) and 100,000/cm² (with daily medium change). They have a plating efficiency of 50% at 30 cells/75 cm² dish and are T-antigen negative. The cells were subcultured at 3 × 10⁵ cells per 75 cm² Falcon dish every 3 days.

Gold-plated cover slips were prepared according to the method of Harris (18) by evaporating 100 mg of gold in a Denton DV-502 evaporator (Denton Vacuum, Inc., Cherry Hill, N. J.) 15 cm away from glass cover slips carrying an electron microscope copper grid as template for the gold-free areas during the evaporation. We used circular cover slips (Kimble Exax, distributed by SGA Scientific, Bloomfield, N.J.) with a diameter of 12 mm and a thickness of 0.17 mm. The estimated thickness of the gold-plating is 100 Å. The gold-plated areas were sintered by heating the cover slip over an ethanol flame

for 10 s to prevent the removal of the gold-coat in aqueous media.

Scanning electron microscopy is described elsewhere (4). Experimental cells from 24-h old cultures were suspended in phosphate-buffered saline solution containing 0.5 mM ethylenediaminetetraacetic acid and 0.05% trypsin. Equal amounts of complete medium were added, and the cells were plated on cover slips in 2.5-cm Falcon plastic dishes (Falcon Plastics, Division of Bioquest, Oxnard, Calif.) which contained fresh medium. For observations on living cells, cells were allowed to attach to glass cover slips for 10 min. Then the cover slip was inverted and put on a microscope slide with pieces of another cover slip as spacers. The space between cover slip and slide was filled with the same medium in which the cells had been plated, and the edges of the cover slip were sealed with wax. During the observation, the microscope stage was kept at 34° ± 3°C by an airstream incubator.

Micrographs of fixed cells were taken after 60-min fixation in 1% glutaraldehyde in phosphate-buffered saline, subsequent incubation for 30 min in distilled water, and embedding in Elvanol (DuPont Instruments, Wilmington, Del.). Pictures were taken in a Zeiss Photomicroscope II on Kodak Panatomic X film.

Chemicals

Cytochalasin B (9) was purchased from Aldrich Chemical Co., Inc., Cedar Knolls, N.J., and kept in frozen stocks of dimethyl sulfoxide at a concentration of 1 mg/ml. The experimental concentrations were obtained by diluting the stock solutions immediately before the experiment.

RESULTS

Filopodia-Mediated Reaction of Spreading 3T3 Cells on Glass to an Adjacent Gold Substratum

Figs. 1 a and b show the characteristic scanning motion of free filopodia. During this lateral wav-ing, the filopodia appear as rigid rods which do not bend despite their rapid movement in the rather viscous culture medium. A similar movement has been described for microspikes at the growth cone of neurites (32).

We considered filopodia to be attached to the substrate when they stopped their scanning motion and remained in their position for more than 1 min. We observed freshly plated 3T3 cells which had settled on glass and whose filopodia contacted both glass and an area of evaporated gold nearby. The technique of evaporating metal onto a nonmetallic substratum through the openings of a template was introduced by Carter (7) and Harris (18). The technique requires a short sintering process to prevent the detachment of the metal coats in aqueous media. Using scanning electron microscopy, we found that sintering of gold coats on glass cover slips resulted in the formation of small metal particles of about 0.3 μm diameter.

Observations on living cells showed a preferential extension of lamellipodia towards gold areas which was preceded by a contact of filopodia with the gold (Fig. 2 a–d). Even though filopodia had contacted both glass and gold, the lamellipodia extended towards gold 4–6 min after the filopodium contact. Lamellipodia could also extend towards the glass substratum, but this occurred 5–10 min later. We found this behavior of freshly plated 3T3 cells in 10 cases out of 12 time-lapse observations of single cells. In one of the 10 cases, the first lamellipodium retracted after an initial extension towards the gold area. Measuring the distance between the cell body and the gold area, and the time between the appearance of a lamellipodium and its first reaching the gold area, we found an average displacement of lamellar extension of 1.3 (SD 0.4) μm/min (10 observations).
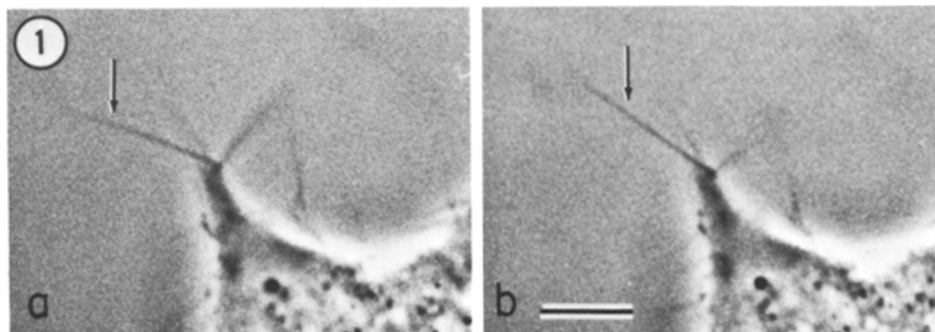

FIGURE 1 (a and b) Scanning motion of rigid filopodia 40 min after plating. Time difference between (a) and (b) is about 10 s. Bar indicates 5 μm.
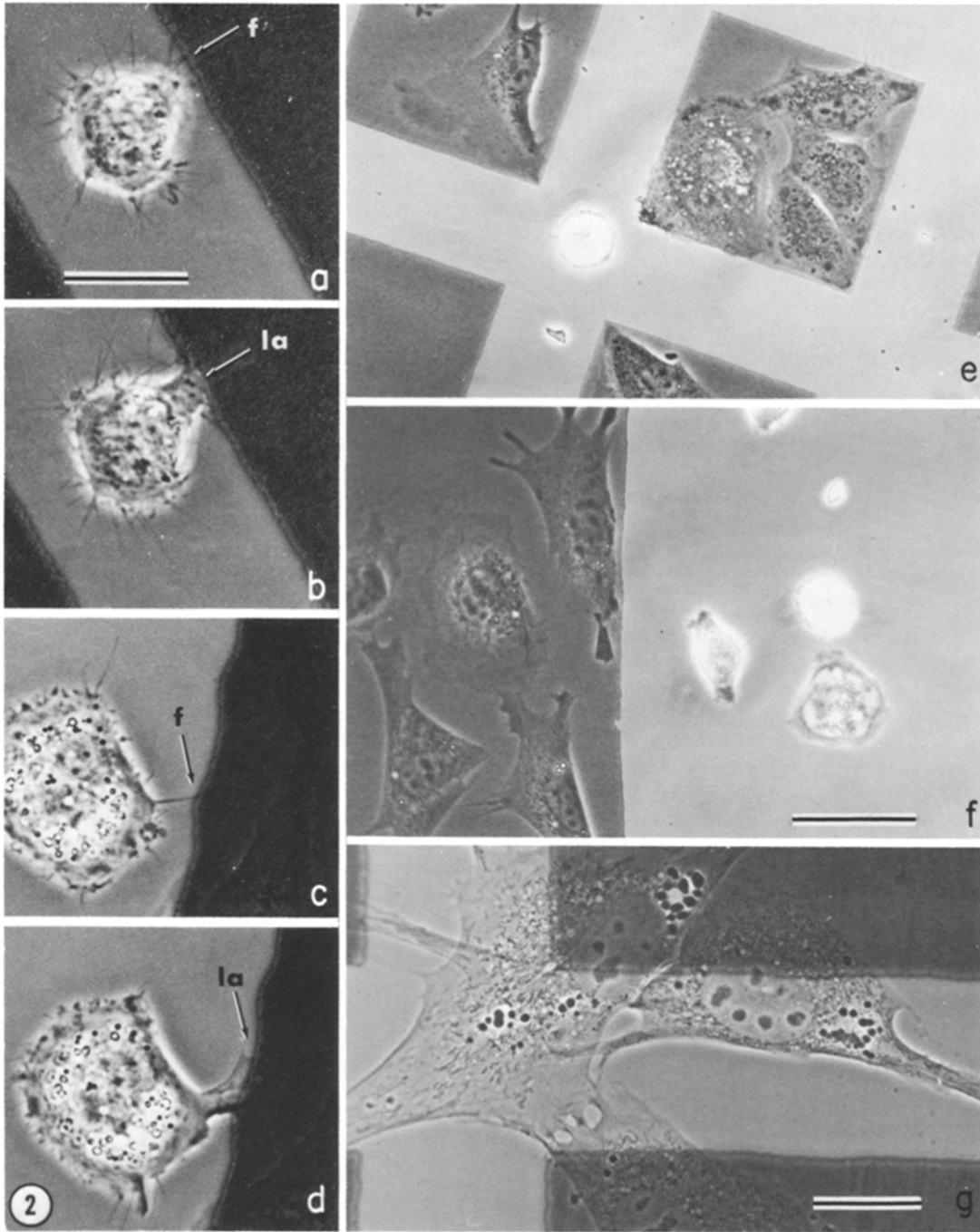
FIGURE 2   Spreading of a freshly plated 3T3 cell near the borderline between gold (dark) and glass (bright). Bar indicates 20 μm. (a) t = 16 min after plating. Filopodia (f) grow out to all sides and establish contact with gold-plated area. (b) t = 20 min. Lamelliopodia (la) are flown out to the gold side and are being filled with cytoplasma. (c) Another 3T3 cell at t = 62 min. Filopodium contact to the gold-plated area has been established. (d) t = 68 min. New lamellipodia flow over to the gold side. (e) Crowding of 3T3 cells in gold-plated areas 3.5 h after plating. (f) Delay in spreading of 3T3 cells on glass compared to gold 3.5 h after plating. Bar indicates 50 μm. (g) Loss of preference for gold-plated areas by 3T3 cells 24 h after plating. Bar indicates 20 μm.

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This value is within the reported range of the net displacement of the leading edge of fibroblasts and epithelial cells. Abercrombie (3) measured, as net displacement of the leading edge, 0.6 (SD 0.3) μm/min for chick heart fibroblasts and 0.7 (SD 0.6) μm/min for mouse muscle fibroblast-like cells. DiPasquale (13) reported 1.7 (SD 1.6) μm/min for normal gut epithelial cells. Both authors observed a sequence of protrusions and withdrawals during the net extension of the leading edge. In contrast to this movement of the leading edge, the extension of lamellipodia along gold-attached filopodia seemed to be a largely constant movement towards the gold area.

1-2 h after plating, we found 80 ± 5% of the cells within the gold squares, although the area of the squares represented 50% of the totally available area. Between 2 and 6 h after plating, most of the cells remained inside the gold-plated areas (Fig. 2 e). After 9 h, only 65% of the cells were left in the gold-plated areas. At 24 h after plating, no preference of the cells for gold-plated areas was visible (Fig. 2 g). If fresh cells were plated at this time in addition to the fully spread cells, the new cells spread symmetrically without reacting differently to the two substrata. A serum-coat or a microexudate carpet which had been formed during 24 h may be the reason why the difference in the adhesiveness between gold and glass disappeared.

In two cases, we observed by time-lapse microphotography how the spreading 3T3 cells moved from their original attachment spot on glass to the gold area. In these cases, after an initial filopodium contact the first lamellipodium extended to the gold area, then new filopodia appeared at the front of the lamellipodium, followed by new lamellipodia which further invaded the gold area. During this process, filopodia and lamellipodia also appeared on the glass side of the cell but the lamellipodia retreated again. During this process, the outlines of the cells remained roughly circular although the cells grew in diameter as they gradually flattened. We measured for 1 h the net displacements of the proximal and distal cell edges with respect to the gold-glass borderline. The arithmetical mean of these two displacements may be considered as the net displacement of the cell. We found a displacement of the cells of about 0.2 μm/min, which was directed to the gold area.

We found that filopodia were produced around the entire perimeter of the spreading cell, no matter whether the cell had attached to glass, to gold, or to the borderline between glass and gold. Cells on glass and at the borderline between glass and gold were delayed in flattening compared to cells on gold (Fig. 2 f; cells a and c in Fig. 3 A). If lamellipodia preferentially extended towards a gold area, they seemed to be actively ruffling (cell b in Fig. 3 B).

We have reported earlier (4) that CB reversibly inhibits the removal of gold particles around a spreading 3T3 cell. Under normal conditions, the particles are removed by filopodia which seem to exert a retraction force upon contact with the particle. Studying the effect of CB on the preferential extension of lamellae towards a gold area on glass, we found a reversible inhibition of this phenomenon, too (Fig. 4). Studies on living cells confirmed that the filopodia cannot retract in the presence of 5 μg/ml CB and often accumulate in number, as indicated in Fig. 4 b. Within 5-10 min after the removal of CB-containing medium, the preferential extension of lamellipodia could be observed again.

Filopodia-Mediated Reaction of a Spreading 3T3 Cell to a Neighboring Spread-Out 3T3 Cell

Filopodia at the growth cone of neurites seem to be able to direct a growing neurite away from another one (14). We tested to determine whether a similar effect could be found in spreading 3T3 cells. Fig. 5 shows a freshly plated 3T3 cell contacting a spread 3T3 cell, which was plated out 24 h before on a glass cover slip. Even though filopodia have grown out all around the fresh cell and contacted both the glass and the already spread cell (Fig. 5 a), the first lamellipodia extended away from the spread cell (Fig. 5 b–d). In Fig. 5 d, a small lamella is formed at the contact area between the cells. Direct observation gave the impression that the lamella was formed by the spread-out cell. Fig. 5 e shows another case in which such a formation did not occur at the contact area. Therefore, it may be possible that sometimes even the spread-out cell reacts to the contact of the filopodia of another cell under certain conditions which are as yet unknown. The segment of the perimeter of the spreading cell that is directed away from the already spread cell is, of course, larger than the segment of the perimeter that is close to the spread cell. Therefore, one expects statistically a larger number of lamelli-
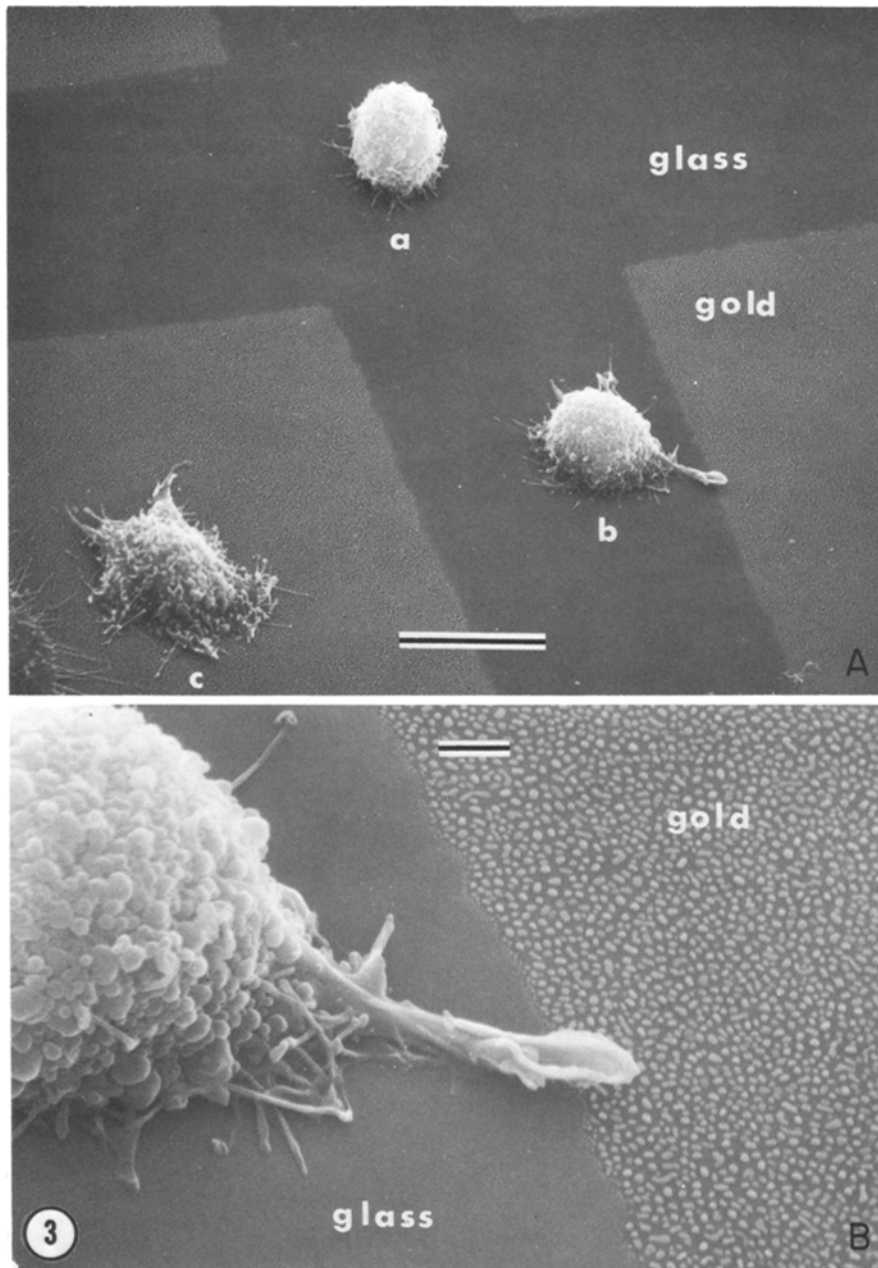
FIGURE 3 Spreading of 3T3 cells on a glass substratum which is partially coated with evaporated and sintered gold. Scanning electron microscopy. Tilt angle: 45°. (A) Different degrees of cell spreading 30 min after plating: cell (a) is sitting on glass and poorly spread; cell (b) has formed a lamellipodium which reaches over to the gold area; cell (c) is well spread on the gold area. Bar indicates 20 μm. (B) Cell (b) at higher magnification showing that the extending lamellipodium is ruffling. Note the granulated structure of the evaporated gold due to the sintering process. Bar indicates 2 μm.
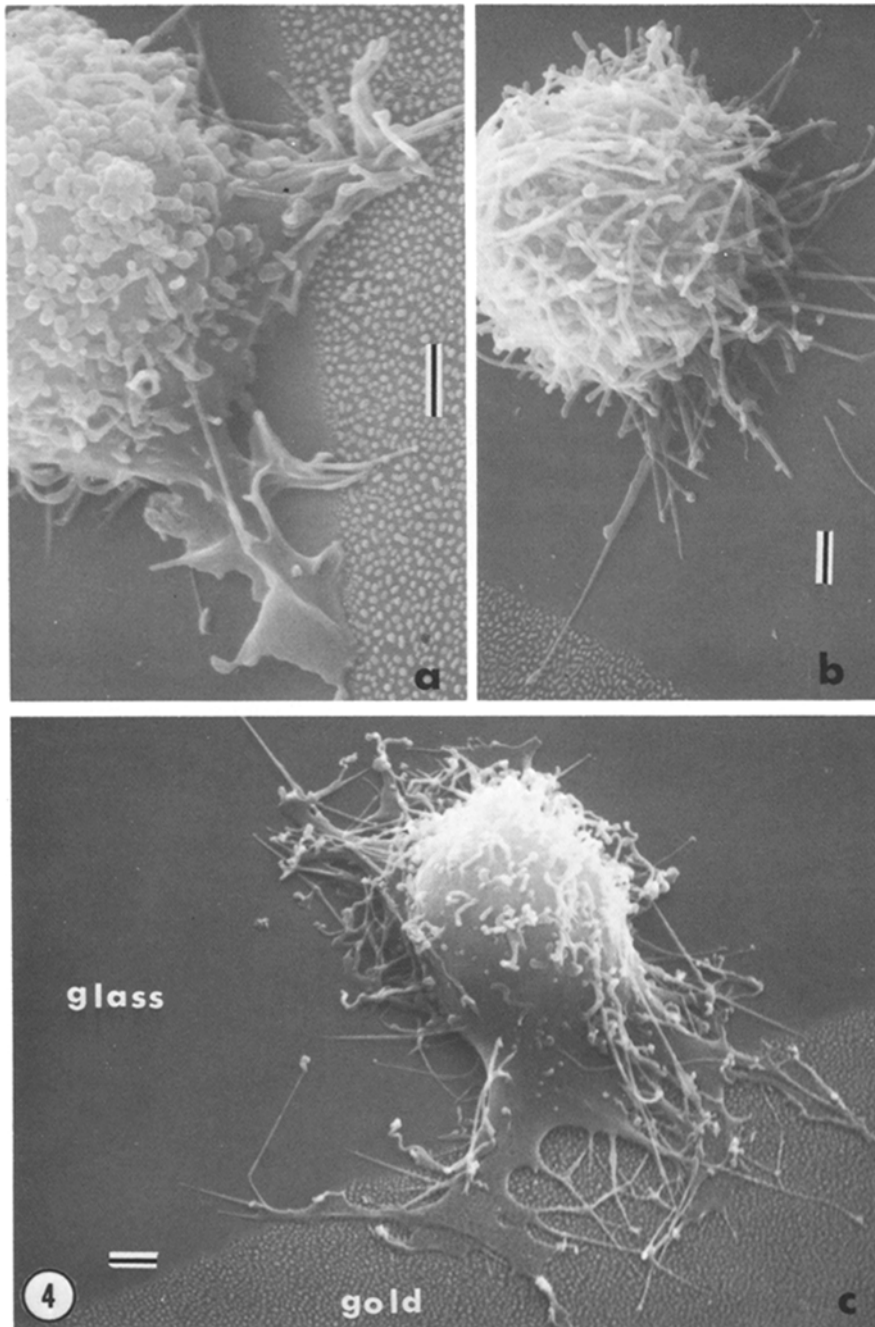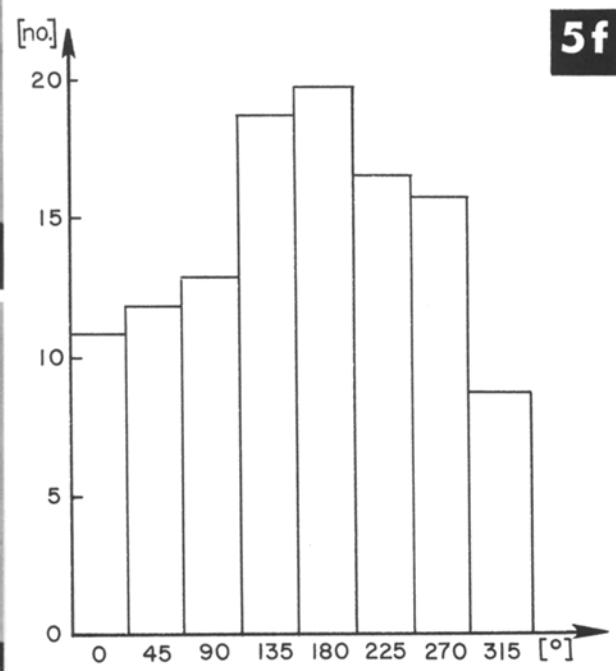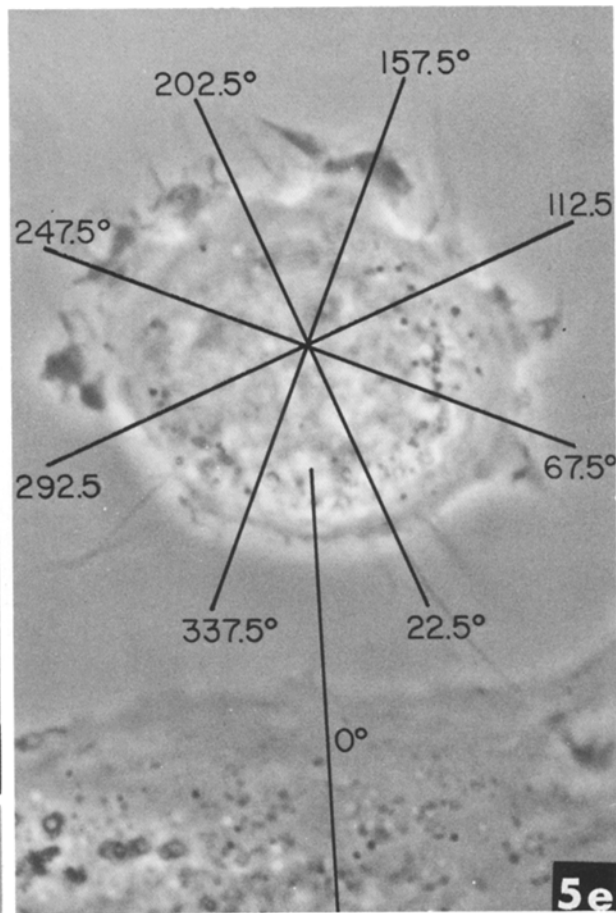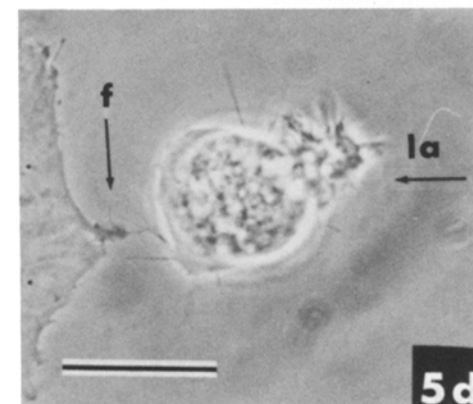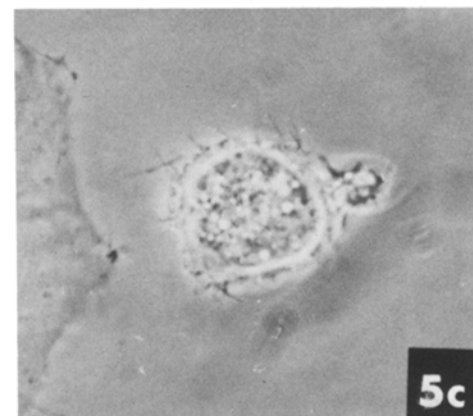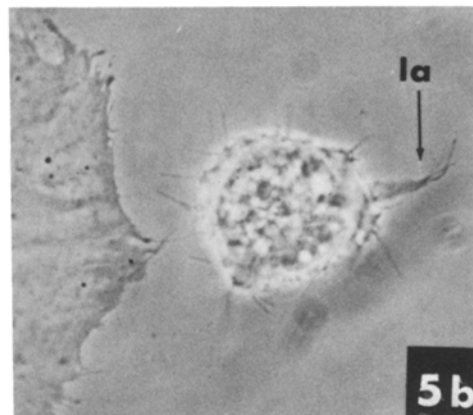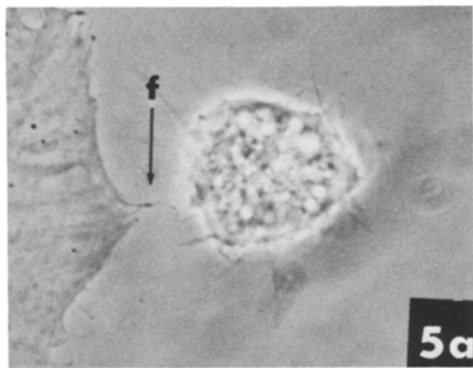
FIGURE 4 Reversible inhibition by CB of the "recognition" of the gold area by spreading 3T3 cells near the glass-gold borderline in scanning electron microscopy. Tilt angle: 45°. Bars indicate 2 μm. (a) Cell 30 min after plating in normal medium. A ruffling lamellipodium extends towards the gold area. The dorsal surface is covered with small blebs. (b) Cell 50 min after plating in medium containing 5 μg/ml CB. The cell is covered with long microvilli and no lamellipodia have extended. (c) Cell 45 min after plating in medium containing 5 μg/ml CB followed by 5 min in normal medium. Lamellipoidia have extended to the gold area, and the cell surface shows only a few microvilli and, again, small blebs.

5a

5b

5c

5d

5e

5f

202.5°   157.5°

247.5°   112.5

67.5°

292.5

337.5°   22.5°

0°

[no.]

20

15

10

5

0

0 45 90 135 180 225 270 315 [°]

282

podia extending away from the spread cell than towards it, even if the direction of the lamellar extension is completely random. In order to eliminate this trivial explanation of the results, we divided the perimeter of the round cell into equal sectors of 45°. The obtained polar coordinate system was oriented such that the center of one sector (−22.5° to +22.5°) included the shortest distance to the already spread cell which was contacted by filopodia (Fig. 5 e). In 39 single cell observations, we counted 117 lamellipodia according to the sector into which they extended. Fig. 5 f shows in a histogram that the largest number of lamellipodia extended to the side away from the already spread cell.

In order to test the effect of CB on this phenomenon, we plated 3T3 cells on glass cover slips in normal medium and allowed them to spread for 24 h. Subsequently, the medium was replaced by medium containing 5 µg/ml CB. Fresh 3T3 cells (which also had been in another culture for 24 h) were plated at the same time. During the next 40 min, the fresh cells attached and sent out filopodia without producing lamellipodia, while the already spread cells assumed their typical CB-induced arborized shape (9, 19, 24), with processes full of zeiotic vesicles (16) (Fig. 6). After 40 min, the CB-containing medium was replaced by fresh medium in order to study the first reactions of the freshly plated cells to the now arborized and 24-h older cells. Fig. 6 shows that again the first lamellae of the freshly plated cell extended away from the already spread cell, while the already spread cell began to resume its normal morphology upon the removal of CB from the medium. Both effects occurred rapidly. We observed this phenomenon in four experiments.

DISCUSSION

We consider 3T3 cells a convenient model system for studying a possible exploratory function of filopodia. Our results seem to suggest such a function, but they do not exclude that filopodia fulfill other functions as well (30). From a morpho-

logical point of view, it seems to be easy to define filopodia. However, the rapid morphological changes which occur in living cells may eventually render a distinction between filopodia, lamellipodia, and blebs arbitrary. Yet, for the time being, it seems practical to consider filopodia as distinctive cellular organs. Future investigations will have to show whether the function of filopodia can also be expressed in other cellular extensions, such as blebs or ruffling edges, and whether it is suppressed in certain cell lines.

In agreement with Carter (8), we interpret the attachment of filopodia to be the result of physico-chemical adhesion between the substratum and the filopodium surface. The same adhesion is presumably also important in order to retain the extended lamellipodia in an advanced position. However, the fact that filopodia grow symmetrically out of cells which are attached to glass, to gold, or to the borderline between the two substrates indicates that their production is cellular- and not substrate-dependent.

Spreading 3T3 cells at the borderline between gold and glass extend their first lamellipodia in the direction of the gold. This reaction is apparently mediated by filopodium contact, because only filopodia and not the cell body are in contact with the other substratum at the time of the lamellar extension. There seems to be a rather trivial explanation for these findings: once the tip of a filopodium has contacted an area of high adhesion, membrane material simply might be sucked over to this area by physico-chemical "wetting forces." However, such an explanation would predict that the lamellipodia creep out of the filopodium tip in circular fashion, in contrast to our observation that the lamellae advance from the cell body and are often actively ruffling. Therefore, the preceding findings seem to us to strongly suggest the exploratory function of filopodia.

This interpretation is supported by the observation that the first lamellipodia of a freshly plated 3T3 cell extend away from a neighboring spread 3T3 cell. This behavior suggests that filopodia may

FIGURE 5 Reaction of a spreading 3T3 cell to the contact by filopodia with another 3T3 cell, which was plated out 24 h before. Bar indicates 20 µm. (a) t = 32 min. The first filopodia make contact (arrow). (b) t = 39 min. The first lamella extends to the side away from the spread cell (arrow). (c) t = 43 min. (d) t = 47 min. (e) Orientation of a polar coordinate system in order to determine the angular distribution of extending lamellipodia after filopodium contact with another already spread 3T3 cell. (f) Histogram of the number of lamellipodia extending into the respective sectors. abscissa: angular interval. ordinate: number of observed lamellae.
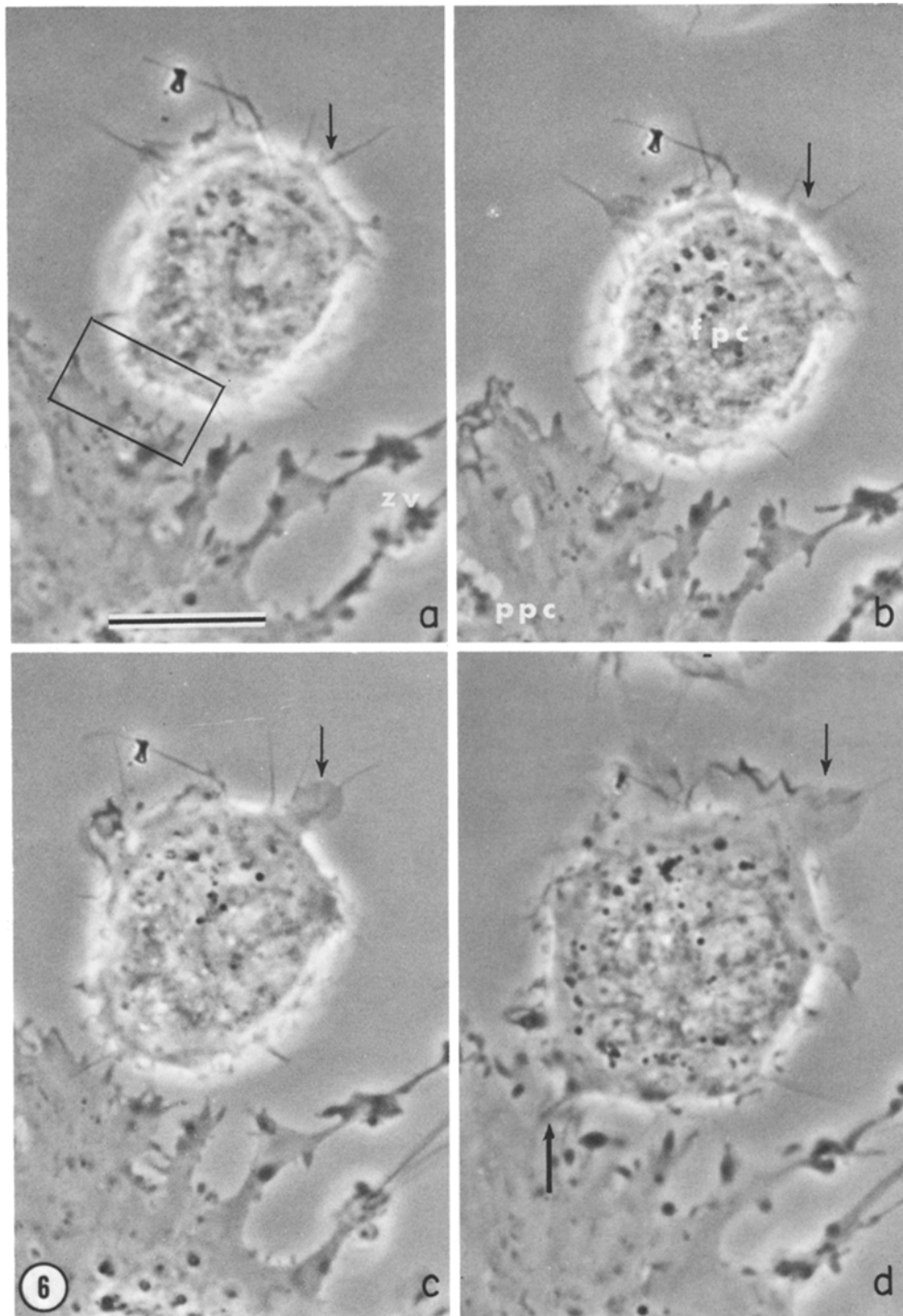
FIGURE 6   Restoration of the recognition of another spread-out cell after removal of CB from the medium, in living cells. Phase-contrast microscopy. Bar indicates 10 μm. Both the previously plated cells (*ppc*) and the freshly plated cells (*fpc*) had been in medium containing 5 μg/ml CB for 40 min. (*a*) 8 min 20 s after removal of CB. Arrow points to one of the first lamellipodia of the fpc which flow to the side away from the ppc, which has assumed the typical arborized shape. The frame shows the contact area of filopodia. (*b*) 8 min 30 s after removal of CB. (*c*) 9 min after removal of CB. (*d*) 10 min 30 s after removal of CB. The ppc has resumed more of its normal shape and seems to form a groove around one of the processes of the fpc (arrow). *Zv* means zeiotic vesicle.

be involved in the attempt of a spreading cell to avoid overlapping with another spread cell. At later stages of spreading, lamellipodia will form all around the perimeter of the cell, and some of them will grow into larger cell processes. Therefore, the final shape and position of the spread cell is hardly determined alone by the first filopodium contact of a still rounded cell. Yet, this first contact could be one among other factors which contribute to a successful reduction in the overlapping with other cells. It must be pointed out, however, that the latter results are not unambiguous as to the role of filopodia in the observed directionally differentiated extension of lamellipodia. Unlike the situation in the experiments on gold-plated glass substrates, we do not know the exact quality of a substrate in the very near vicinity of a spread-out cell. It may be that a short-range gradient of a cell-mediated coat on the substrate or of a cell-mediated compound in the fluid environment of the spread cell causes the spreading cell to extend directly lamellipodia away from the spread-out cell without involving filopodia at all. On the other hand, we cannot exclude either that those extracellular parameters, if present, are "detected" by the filopodia of the spreading cell.

If we assume that the adhesion between two 3T3 cells is weaker or faster than cell-glass adhesion (1), and that cell-glass adhesion is weaker or faster than cell-gold adhesion, both the lamellar extension towards a gold area and the extension away from an already spread 3T3 cell would be consistent with the following interpretation: filopodia which have grown out all around a freshly plated cell initiate lamellar extension towards their attached tip the faster, the firmer or faster the tip is attached. In this way, lamellipodia extend towards glass before they extend towards another cell, or earlier towards gold than towards glass.

As to the mechanism of the exploratory function of filopodia, the production of a retraction force upon attachment may be of central importance. The removal of particles around a spreading 3T3 cell may reveal that filopodia always exert a retraction force if they attach to the surface of another object, be it a particle, an extended solid substratum, or another cell. Freshly plated 3T3 cells on particle-coated cover slips showed, in addition, that lamellar extensions did not occur until the loose particles around the cells had been removed within a radius of about 20 μm (4). This observation may indicate that lamellar extension is not initiated if such a retraction force leads to an actual shortening of the whole filopodium, i.e.,

lamellar extension requires an isometric retraction of the filopodium. In a perhaps more illustrative way, one might say that a cell tests the firmness of a filopodium contact before it extends a lamellipodium, by testing whether the retraction force will break the contact and retract the filopodium as a whole. Observations on the normal spreading of living 3T3 cells on glass confirm that filopodia attach to glass and remain attached before any lamellipodia extend. Although retraction fibers (23) of mitotic cells or of cells at low trypsin concentrations may be different from filopodia, they seem morphologically identical to attached filopodia. They also remain attached in a stretched-out state before lamellipodia re-extend along them.

The importance of the retraction force for lamellar extension seems to be emphasized also by the results of the experiments with CB. At the same concentrations at which CB inhibits the retraction of filopodia as assayed by particle removal, the preferential extension of lamellipodia towards a gold-plated area or away from another spread cell is reversibly inhibited, too. There is, of course, the alternative possibility that CB inhibits the formation of lamellipodia (9) in addition to the production of a retraction force. We cannot exclude this possibility. However, our reported correlation between inhibition of particle removal and lamellar extension by CB opens the possibility that inhibition of lamellar extension by CB is preceded by an inhibition of production of force by filopodia.

The mechanism of production of force is presently unknown. At present, we cannot decide whether an actomyosin sliding filament model can explain the production of force, although Bray's model (6), with minor modifications, can account for several phenomena of movement which we observed with filopodia of spreading 3T3 cells. Also, the role of cyclic AMP in the retraction of filopodia, as suggested by Willingham and Pastan (33) for the growth of filopodia, awaits further experimentation.

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