A QUANTITATIVE DESCRIPTION OF THE EXTENSION AND RETRACTION OF SURFACE PROTRUSIONS IN SPREADING 3T3 MOUSE FIBROBLASTS

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ABSTRACT

We suggest a method of quantitating the motile actions of surface protrusions in spreading animal cells in culture. Its basis is the determination of the percentage of freshly plated cells which produce particle-free areas around them on a gold particle-coated glass cover slip within 50 min. Studying 3T3 cells with this assay, we found that the presence of Na⁺, K⁺, Cl⁻, and Mg²⁺ or Ca²⁺ in a neutral or slightly alkaline phosphate or bicarbonate buffered solution is sufficient to support the optimal particle removal by the cells for at least 50 min.

Two metabolic inhibitors, 2,4-dinitrophenol and Na-azide, inhibit the particle removal. If D-glucose is added along with the inhibitors, particle removal can be restored, whereas the addition of three glucose analogues which are generally believed to be nonmetabolizable cannot restore the activity.

Serum is not required for the mechanism(s) of the motile actions of surface protrusions in spreading 3T3 cells. However, it contains components which can neutralize the inhibitory actions of bovine serum albumin and several amino acids, particularly L-cystine or L-cystein and L-methionine. Furthermore, serum codetermines which of the major surface extensions, filopodia, lamellipodia, or lobopodia, is predominantly active.

We found three distinct classes of extracellular conditions under which the active surface projections are predominantly either lamellipodia, (sheetlike projections), lobopodia (blebs), or filopodia (microspikes). The quantitated dependencies on temperature, pH and the inhibition by cytochalasin B of the particle removal are very similar in all three cases. Preventing the cells from anchoring themselves for 15–20 min before plating in serum-free medium seems to stimulate particle removal threefold.

As long as we do not fully understand the logic and mechanisms underlying the phenomena of animal cell motility, they will continue to appear polymorphous and erratic. A necessary first step toward such understanding is the development of quantitative assays for the phenomena of animal cell motility. The locomotion, i.e., the displacement of whole cells on an artificial substrate, has been successfully quantitated (12, 17), as have the translocational and rotational movements of single molecules within the plasma membrane (13, 22, 26), the collective movement of many antigenic sites on the cell surface, i.e., the capping phenomenon (16, 34), and the movement of single, sur-
Surface-attached particles (7, 19). However, a quantitative assay for the motility phenomena which are associated with the large cell surface extensions, i.e., lamellipodia, lobopodia (blebs), and filopodia (microspikes), has not yet been described.

Lamellipodia (1, 2) are sheetlike cell projections usually extending parallel to the substrate. Blebs are hemispherical extensions which can cover a whole cell surface like bubbles. If they extend farther out, changing to a "sausage"-like morphology, they are called lobopodia (32). In this paper, we will not distinguish between blebs and lobopodia. Filopodia (31) or microspikes (29) are needlelike projections approx. 0.2 μm in diameter and varying in length between 5 and 30 μm. In still photographs, they are not distinguishable from retraction fibers (25). The distinction is made in live-cell observations, where retraction fibers are seen to be "left-overs" from a retracting lamellipodium or lobopodium and are attached to the substrate, whereas filopodia extend actively from the cell surface and can move freely until they attach. Filopodia or retraction fibers are distinct from microvilli. These are cylindrical projections approx. 0.1-μm thick and only up to 2-μm long which can cover whole cell surfaces like "fur." Because of their size, we cannot observe their motile actions during cell spreading in light microscopy. They will not be considered in this paper.

None of these projections is a permanent surface specialization in cultured fibroblasts. Dynamic transitions within seconds or minutes from one type to any other can be observed in live cells, and this contributes considerably to the difficulty of quantitating their actions.

The literature includes several careful descriptions of the motile behavior of these cell projections, and quantities such as extension speed or withdrawal frequency have been determined (1, 2, 14, 15). Such quantitation, however, is extremely time consuming, is inevitably derived from only a small number of cells, and is hampered by possible morphological changes of the projections during observation.

In approaching the problem of quantitating the phenomena of the motile actions of surface protrusions within a large number of cells, one may try to examine the occurrence of a fundamental event which is likely to underlie the polymorphous expressions of the phenomena and which is accessible to quantitation. We suggest choosing for this purpose the following three-step event: (a) extension of a cellular projection, (b) attachment of the projection to an object at some distance from the cell body, and (c) production of a force which tends to shorten the distance between attachment spot and cell body.

We shall call this three-step event an "extension-retraction cycle" as a generalization of the "ruffling cycle" (23).

In order to quantitate the capacity of cells to perform extension-retraction cycles, we took up an observation which was made by several investigators (3, 14, 19, 23). Small particles placed on a cell substrate can be picked up by cells and transported to the perinuclear region. We found that gold particles, 0.2-0.4 μm in diameter, coat a glass cover slip very densely and evenly. If suspended cells are plated on such a coated substrate, the particles are removed around 80-90% of the cells within 30-60 min. Easily identifiable rings can be seen (see Fig. 1 b) which have twice the diameter of the cell bodies, with most of the cells still being spherical (6). Live-cell studies confirmed the conclusion suggested by this observation. The cells extend projections which reach as far as the perimeter of the rings, attach to the loose particles, and transport them to the cell body either by a centripetal flow mechanism or by retraction as a whole together with the particle. In other words, they perform extension-retraction cycles.

Making use of this phenomenon under standardized and experimentally controlled conditions, one can readily quantitate the capacity of cell projections to perform extension-retraction cycles by determining the percentage of cells that produce particle-free rings. It may be possible to improve the quantitation by measuring the sizes of the cleared areas around the cells, in addition. At present, however, we prefer to merely quantitate the capacity of cells to extend and retract surface projections. In this paper, we present the results of such quantitation as applied to 3T3 mouse fibroblasts.

MATERIALS AND METHODS

Cell Cultures

3T3 cells (30) were a kind gift of Dr. H. Green, Massachusetts Institute of Technology, Boston, Mass. The cells were grown in Dulbecco's modification of Eagle's medium (DME) from Grand Island Biological Co., Grand Island, N. Y., supplemented with 10% calf serum (Microbiological Associates, Bethesda, Md.), at 37°C in
of Data

Inoculation of Cells and Evaluation

A 9-cm Falcon plastic dish containing cells plated 48 h earlier was washed twice in 0.5 mM EDTA + 0.05% trypsin in PBS. The suspension solution was removed except for a thin fluid film on top of the cells, and left for 3 min in the incubator. The rounded-up cells were then gently washed off the dish in 6 ml of medium with 10% serum, spun at room temperature for 3 min at 1,000 rpm in a desk centrifuge, and resuspended in new medium. This procedure, which takes about 4 min, will be referred to as a 1 × wash. Larger numbers, e.g., 5 ×, will indicate further repetition of centrifugation and resuspension in fresh solution. Approx. 3 × 10^4 suspended cells were added to each of the 3.5-cm Falcon dishes containing the gold-coated cover slips, and these were left in the incubator at 37°C for 50 min, unless otherwise stated.

Figs. 1a and b illustrate the procedure of counting cells with and without rings of removed particles. In our experience, the decision whether or not a cell has removed particles is hardly ever questionable. Typically, for one experimental point, we counted about 150 cells on each of the two cover slips. Unless otherwise stated, the reported data are derived from 2-11 independent experiments. If the described procedure is carefully observed, the numerical data are reproducible within ±8% removal, e.g., a value of 40% removal in any of the graphs means 40 ± 8%. Exposure of the cells to the gold particles has no obvious toxic effects. We kept cells for 1 wk on a gold-coated cover slip, treated them with trypsin, and plated them on another. After another week, we fixed the cells and prepared them for microscopy. Fig. 1c–e shows these cells. They had ingested vast amounts of particles and yet appeared reasonably normal. We also found normal growth and monolayering of 3T3 cells on gold-coated cover slips.

The kinetics of particle removal in normal culture medium with 10% calf serum is shown in Fig. 2. 40 min after plating, a plateau is reached. At later times, the cells begin to spread into the cleaned areas.

Gold Particle Preparation

This has been described elsewhere (6). We found the use of Na₂CO₃ more convenient than that of K₂CO₃ and changed the procedure accordingly.

Gold Coating

Circular 12-mm glass cover slips (Kimble, distributed by SGA Scientific, Bloomfield, N. J.) were dipped into an aqueous solution of 10 mg/ml bovine serum albumin (BSA) at room temperature (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.), quickly drained over the edge on a paper towel, dipped in 100% ethanol at room temperature, again quickly drained on a paper towel and placed parallel to an 85°C airstream from a hairdryer. The cover slip should usually be dried within 10-15 s without showing any light-scattering deposits. The cover slips were placed singly in the wells of a 24-well Linbro disposable tray (Model FB16-24TC, Linbro Chemical Co., New Haven, Conn.), and 1 ml of the gold suspension (60-176 was added. After 45 min of incubation in the gold suspension, the cover slips were washed in normal salt solution (see below), then drained, and two cover slips were placed into a 3.5-cm Falcon plastic dish (Falcon Plastics, Division of BioQuest, Oxford, Calif.) containing 2 ml of the experimental medium. These test dishes were kept on a 37°C warm plate until needed for the inoculation of cells.

Inoculation of Cells and Evaluation of Data

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Figs. 1a and b illustrate the procedure of counting cells with and without rings of removed particles. In our experience, the decision whether or not a cell has removed particles is hardly ever questionable. Typically, for one experimental point, we counted about 150 cells on each of the two cover slips. Unless otherwise stated, the reported data are derived from 2-11 independent experiments. If the described procedure is carefully observed, the numerical data are reproducible within ±8% removal, e.g., a value of 40% removal in any of the graphs means 40 ± 8%. Exposure of the cells to the gold particles has no obvious toxic effects. We kept cells for 1 wk on a gold-coated cover slip, treated them with trypsin, and plated them on another. After another week, we fixed the cells and prepared them for microscopy. Fig. 1c–e shows these cells. They had ingested vast amounts of particles and yet appeared reasonably normal. We also found normal growth and monolayering of 3T3 cells on gold-coated cover slips.

Particle-Substrate Adhesion

The BSA-treatment of the glass surfaces is necessary to prevent detachment of the particles upon incubation in serum-free media. After the ethanol fixation and heat drying, the BSA is denatured and seems to form a thin film on the glass surface to which the particles can stick with a reproducible strength. Denaturation of the BSA is important because undenatured BSA completely inhibits particle removal in concentrations of 1 mg/ml. We measured the adhesion force between particles and substrate under standardized conditions in Dulbecco’s modification of Eagle’s medium with 10% calf serum (DME + CS) at 25°C by centrifugation of particle-coated cover slips for 5 min at various g-values. For vertical detachment of 50% of the particles, we found a g-value of 4,500 g, for sliding 1,500 g. Assuming an average cluster of gold particles of 1 μm diam, the values correspond to forces of 4.8 × 10^-4 dyn and 1.6 × 10^-4 dyn, respectively.

Solutions

NORMAL SALT SOLUTION: 113 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 15 mM Na-phosphate, 15 mg/ml phenol red, pH 7.8 in quartz-distilled water.
WASHING SOLUTION: 140 mM NaCl, 3 mM KCl in quartz-distilled water.

Chemicals

The chemicals used were purchased from the following companies: Aldrich Chemical Co., New Jersey (cytochalasin B); J. T. Baker Chemical Co., Phillipsburg, N. J. (Na₂SO₄, NaHCO₃, MgCl₂, CaCl₂, and d-glucose); Eastman Kodak Co., Rochester, N. Y. (Na-azide); Mallinckrodt Chemical Works, St. Louis, Mo. (Na-pyruvate, NaCl, KCl, and Fe (NO₃)₃); Merck Chemical Div.,
FIGURE 1 (a and b) Particle removal on gold particle-coated glass cover slips by 3T3 cells 50 min after plating. Phase-contrast image of a field for counting the total number of cells (a). Bright-field illumination of the same field (b). The cells become invisible, and the number of cells which removed the particles is determined by counting the number of particle-free rings. Arrows point to cells which removed particles. Bar indicates 50 μm. (c-e) Nontoxicity of the particles for 3T3 cells. (c) Differential interference contrast (Nomarski) image of cells which have been for 2 wk on particle-coated cover slips. The black areas are accumulations of particles. The space between cells has been completely cleaned of particles. Bar indicates 50 μm. (d) Scanning electron micrograph of the same preparation to show the internalization of the particles which are represented by the blurred grey clusters, in contrast to an extracellularly located particle cluster (arrow) which is bright white. Density and distribution of microvilli (mv) seems normal. Bar indicates 2 μm; tilt angle 45°. (e) Whole cell in scanning electron microscopy to show that the nuclear region is free of ingested particles (likewise in panel d). Bar indicates 10 μm; tilt angle 68°.

Merck & Co., Rahway, N. J. (2,4-dinitrophenol [DNP]); Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y. (BSA, crystalized); and Sigma Chemical Co., St. Louis, Mo. (amino acids kit, ouabain, NaH₂PO₄, Na₂HPO₄, dimethylsulfoxide (DMSO), 2-deoxy-glucose, 3-O-methyl-glucoside, α-methyl-glucoside). Cytochalasin B was kept in frozen stock solutions of 1 mg/ml DMSO.

Live-Cell Observations and Scanning Electron Microscopy

These have been described elsewhere (5, 6). However, in the present study we omitted the OsO₄ fixation for scanning electron microscopy in order to reduce cracking of cell junctions during the critical point drying.

RESULTS

Removal under Minimal Extracellular Conditions

Provided the cells were washed five times in washing solution, the presence of Na⁺, K⁺, Cl⁻, and Mg²⁺ or Ca²⁺, and phosphate or bicarbonate in the extracellular medium was sufficient to support particle removal. Maximal removal was
reached at 123 mM Na⁺, which coincided with the point of isosmolarity of 290 mosmol for the whole solution.

The presence of extracellular Ca²⁺ or Mg²⁺ improved particle removal, but was probably not required for 5 × washed cells. We found that Mg²⁺ and Ca²⁺ had similar effects on particle removal. Without the addition of either divalent cation, there was already 45% particle removal which increased to 70-80% by adding 1 mM Ca²⁺ or Mg²⁺. Higher Mg²⁺ or Ca²⁺ concentrations did not further increase the particle removal. We cannot exclude the possibility that particle removal in Ca-Mg-free solutions was due to residual Ca or Mg not removed by the 5 × washing procedure. In several of the following experiments, we chose only Mg²⁺ as the divalent cation in the salt solutions, because this choice avoids precipitation of Ca-phosphate in the salt solutions at higher phosphate concentrations and pH values above 7.4.

The influence of K⁺ and phosphate concentrations on particle removal is shown in Fig. 3. The cells were plated into K⁺-free normal salt solutions with various concentrations of Na-phosphate at pH 7.2. In one set of experiments, 3 mM KCl was added. Fig. 3 shows that a plateau value of 50% removal was reached at 2 mM phosphate. If 3 mM KCl was added, the plateau value rose to 70%. The addition of only 3 mM KCl cannot change the removal value by changing the osmolarity of the solution. Therefore, the data suggest that particle removal mechanism discriminates between extracellular Na⁺ and extracellular K⁺. Fig. 3 shows, furthermore, that neither arsenate nor sulfate could replace the phosphate ions.

We found that phosphate can be replaced by bicarbonate. The substitution of phosphate by bicarbonate in the normal salt solutions is problematic because bicarbonate equilibrates with the atmospheric CO₂ concentration. Thus, both the bicarbonate concentration and the pH value of the test solutions change with time. The results on 5 × washed cells plotted as a function of the originally added NaHCO₃ concentration showed that a plateau value of 70-80% was reached above 3 mM-added bicarbonate. The pH values were measured after the experiment. We found that the pH rose with the added bicarbonate concentration to between 5.8 and 7.5. Plateau values were maintained above pH 7.0.

Both phosphate and bicarbonate are buffers which are compatible with the growth conditions of cells. Therefore, one may suspect that particle removal simply requires a buffered solution with a neutral or slightly alkaline pH value. Indeed, at pH 7.1 and 7.6, removal in tris buffer was similar to removal in phosphate buffer. In N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) buffer, however, particle removal was reduced to less than 4% at pH 7.0, 7.4, 8.0, and 9.1. The finding that at least tris buffer can support particle removal between pH 7.0 and 8.0 encourages the interpretation that particle removal requires simply a buffered solution at neutral or alkaline pH.

![Figure 3](image-url) Dependencies of particle removal on extracellular phosphate, arsenate, and sulfate concentration. Improvement of particle removal by the addition of 3 mM K⁺.
The energy required for particle removal must be supplied by intracellular sources, since the extracellular fluid in these experiments did not contain any nutrients. In order to test whether the cellular energy metabolism has to be active during particle removal, we added various amounts of DNP or Na-azide to normal salt solution. Fig. 4 shows that particle removal of 5 × washed cells was rapidly inhibited in the presence of Na-azide. The results with DNP were very similar. Inhibition of particle removal was complete at a concentration of 0.5 mM DNP. If 4.5 mg/ml D-glucose was added, together with the inhibitors, particle removal proceeded at almost normal levels (Fig. 4). If, instead of D-glucose, 4.5 mg/ml of the glucose analogues, 2-deoxy-D-glucose, α-methyl-D-glucoside, or 3-O-methyl-D-glucoside were added to Na-azide containing normal salt solutions, particle removal remained inhibited. 2-deoxy-D-glucose at a concentration of 4.5 mg/ml inhibited particle removal already by itself. The findings suggest that a continuous energy metabolism is required for particle removal. Furthermore, it seems that the cells can obtain the required energy either from oxidative phosphorylation, using intracellular pools of metabolites, or from glycolysis, if oxidative phosphorylation is inhibited and extracellular D-glucose is provided.

The involvement of Na⁺, K⁺, Mg⁺, and presumably ATP in the particle removal, as shown by the data reported so far, might suggest an involvement of the membrane-located Na-K-ATPase (27) in the phenomenon. Therefore, we added ouabain (28) in concentrations up to 1 mM to normal salt solutions. We found that the values for removal were not different from control values for removal. Therefore, if any cellular Mg-ATPases are involved in particle removal, they are likely to be intracellular.

Since particle removal was originally observed in DME + CS (6), we tested all the components of culture medium by adding them singly to normal salt solution in the concentrations in which they are contained in DME (see Gibco catalog). Most of the additions had minimal effects. However, we found that a pronounced reduction of particle removal was caused by the addition of L-cystine (30% removal at concentrations above 5 μg/ml). L-cysteine produced an equal reduction. L-methionine also had an appreciable effect (50% removal of concentrations above 5 μg/ml). All three amino acids caused only partial inhibitions even at concentrations of 100 μg/ml, which could be completely reversed by the addition of 10% calf serum. The data suggest that one of the functions of serum in the motility of surface protrusions under normal culture conditions is to neutralize negative effects of extracellular amino acids, particularly of L-cystine and L-methionine.

We have already mentioned (see Materials and Methods) that our BSA preparation in concentrations of 1 mg/ml inhibits particle removal. Under these conditions, the cell surfaces appear smooth and swollen. Normal calf serum contains about 60% BSA, which corresponds to a concentration of about 4 mg/ml BSA in a normal salt solution to which 10% calf serum has been added. Particle removal was 85% under these conditions. Therefore, we suggest that calf serum also contains rather potent components which neutralize the inhibitory effect of BSA.

Influence of Cell Washing on Particle Removal

In the above described experiments, the cells were washed 5 × (spun down five times, and resuspended in fresh fluid) before being plated into a test solution. The original purpose of this pretreatment was to remove from the cell suspension possible residues of culture medium, serum, trypsin, and EDTA. The experiments described below, however, suggest that the removal of contaminants from the cell suspension is an irrelevant aspect of the washing procedure.

Fig. 5 shows an increase in particle removal with the numbers of washes in various solutions. The decrease in particle removal at washes higher than
seven was accompanied by decreasing cell numbers in the cell suspensions and therefore may be attributed to a presumable loss of viability of the cells. There seem to be three possibilities to explain the rise in particle removal with increasing number of washes. (a) Residual inhibitory components of culture medium, serum, or the trypsin solution may be increasingly diluted in the cell suspension with the successive washes. (b) Exposure of the cells to the mechanical stress of centrifugation and resuspensions may stimulate the motile activity of surface projections, perhaps by removing, from the cell surface, cellular components or poorly soluble, residual and inhibitory components of medium, serum, or the trypsin solution. (c) If cells are kept in suspension, thus being deprived beyond a certain length of time of the opportunity to anchor themselves to a solid substrate, an intracellular process may be stimulated which enhances the motile activity of surface projections of the cells in order to improve their chances of finding anchorage.

To decide between these possibilities, we did the following experiments. Cells were 5 × washed in PBS at pH 7.2 and plated into PBS + 1 mM CaCl₂ + 1 mM MgCl₂ (PBS + Ca + Mg). An aliquot of these cells was resuspended in the supernate of the first wash. If explanation (a) was true, this supernate should have contained the residual soluble contaminants and therefore should have reduced particle removal in PBS + Ca + Mg. We found no reduction of removal compared to 5 × PBS-washed cells.

To test explanation (b), we washed cells 5 × in PBS and kept aliquots of cells from the first wash in suspension by shaking them in PBS for the same length of time (20 min). We used two different shaking frequencies (4 rpm and 250 rpm). The cells were then plated into PBS + Ca + Mg. Although the three procedures exposed the cells to quite different mechanical stresses, particle removal was optimal and equal in all three cases. Therefore, we have to consider explanations (a) and (b) unlikely and are left essentially with explanation (c) or some other possibility.

It seems that the inhibitory components in DME can suppress the stimulatory effect of cell washing. If cells were washed in DME and subsequently plated into DME, particle removal rapidly decreased with increasing number of washes after an initial rise (Fig. 5). The treated cells flattened out on top of the particles without moving them. The neutralizing action of serum seems to be effective also under these conditions, because cells which had been washed for various times in either DME or DME + CS and plated into DME + CS showed maximal levels of removal regardless of the number of washes up to 5 ×. Therefore, particle removal is optimal if serum is present in the plating medium, regardless of the number of washes and of washing medium. In view of interpretation (c) that washing stimulates the motile activity of surface projections by an as yet undetermined intracellular process, the findings suggest that extracellular serum can optimally stimulate these activities as well.
**Morphology of Particle Removal**

Our investigation of the extracellular conditions of particle removal suggested the following rather simple relationships between the predominant involvement of one or the other major cell surface extensions in the particle removal and the extracellular conditions: (a) Absence of serum in both the washing and the plating medium entailed particle removal predominantly by lamellipodia. (b) Addition of serum to normal salt solutions as the plating medium or as the washing and plating medium entailed particle removal predominantly by lobopodia (blebs). (c) DME + CS as both washing and plating medium after only a short exposure to anchorage-free conditions entailed particle removal predominantly by filopodia (microspikes).

**Particle Removal by Lamellipodia:** Fig. 6A shows the morphology of particle removal by cells which had been washed 5 x in washing solution and plated into normal salt solution. We found the starlike appearance of the cells together with somewhat polygonal particle-free areas around the cells (Fig. 6A, section a) to be characteristic for all tested conditions in which serum was absent in both the washing and the plating media. We tested various combinations of normal salt to which the components of DME were added singly, as well as full DME as washing and plating medium. Also, all experiments illustrated in Figs. 3-5 represented conditions in which serum was omitted from both washing and plating media. In all these cases, the removal pattern, if particle removal occurred at all, was indistinguishable from that shown in Fig. 6A, section a. Fig. 6A, section b, shows a typical example of a single cell under those conditions. Thick, spiky processes were invariably seen, producing lamellipodia at their tips and their sides. In live cells, we observed a few growing filopodia, performing their characteristic waving movements. If they became attached to particles, however, they did not remove them. The live-cell studies also showed that the fibrous projections seen in Fig. 6A, section b, were mostly retraction fibers (25) of previously extended lamellipodia. Lobopodia were practically absent in these preparations. The live-cell sequence observed in Fig. 6A, sections c-e, illustrates the way in which the particles were picked up by lamellipodia and transported along the thick processes towards the cell body.

**Particle Removal by Lobopodia:** If the cells were washed 5 x in normal salt or washing solution and plated into normal salt solution or DME to which 10% calf serum had been added, the removal pattern changed completely (Fig. 6B, section a). Almost all the cells remained rounded up and produced circular, particle-free areas. With only few exceptions, the cell surfaces were covered with blebs. Fig. 6B, section b, shows a cell under these conditions. It may be difficult to imagine that lobopodia could extend as far as the perimeter of the particle-free ring in this figure. In live cells, however, several times we observed lobopodia of an extraordinary size which extended within 10-20 s. Furthermore, Fig. 6B, section a, shows that most cells were located off center in the rings, which indicates that the blebbing cells could be displaced during particle removal, thus cleaning an area larger than that corresponding to the maximal reach of lobopodia. Often, growing filopodia preceded the extension of lobopodia, but none of them was observed to remove particles. Short lamellipodia could be seen close to the base of the cell. We observed several more extended lamellipodia which, however, appeared as the result of a retracting and flattening lobopodium. Fig. 6B, section c-d, shows a live-cell sequence of particle removal by lobopodia. One of the photographed particles flowed towards the cell body while the lobopodium remained extended. The other was transported back as the whole lobopodium retracted.

**Particle Removal by Filopodia:** In a previous publication, we have described this mode of particle removal in another 3T3 cell line (6). For reasons of completeness, we repeat it here for the cell line used in the experiments for this paper. If the cells were washed not more than 2 x in DME + CS and plated into the same solution, the removal pattern (Fig. 6C, section a) resembled in some ways the pattern shown in Fig. 6A, section a. The flattened cells were less spiky, however, and the particle-free areas were considerably bigger. The single cell surfaces (Fig. 6C, section b) were covered with microvilli or small blebs, and the cells extended large numbers of filopodia, which, as judged by live-cell observations (Fig. 6C, section c-e), predominantly removed the particles before extension of lamellipodia. Many filopodia were also found to extend from the edge of lamellipodia which had extended after earlier filopodia had removed most of the particles within their reach. Particles which had been missed by the filopodia in the early removal
process, or which were too big or too firmly attached, could be removed by lamellipodia at later stages of spreading. In a few cells, we also found lobopodia. Their number seemed to increase with the number of washes.

Similarities in the Functional Characteristics among Different Modes of Particle Removal

In order to test the dependence of particle removal on pH, temperature, and cytochalasin B concentration, we studied particle removal under each of the following experimental conditions: (a) cells washed 5 x in washing solution and plated into normal salt solution (removal predominantly by lamellipodia), (b) cells washed 5 x in washing solution and plated into normal salt solution with 10% calf serum (removal predominantly by lobopodia), (c) cells washed 1 x in DME + CS and plated into DME + CS (removal predominantly by filopodia). Fig. 7a shows the close similarities in the temperature dependencies with respect to particle removal under the three conditions. Dependence on pH was also very similar (Fig. 7b); however, the curve which describes the predominant removal by filopodia seems to be shifted to more alkaline pH values. For both temperature and pH dependencies, we studied the cell morphology by scanning electron microscopy under condition (c). We did not find any morphological indication that the mode of particle removal changed with different pH or temperature values.

The inhibition of filopodial function by cytochalasin B in medium with 10% calf serum has been described elsewhere (5, 6). Fig. 7c shows the inhibition of particle removal under the conditions (a) and (b), compared to a control containing the appropriate concentrations of DMSO. Fig. 7c shows that the inhibitory activity of cytochalasin B was decreased if serum was present in the extracellular medium.

One may consider the curves of Fig. 7 as showing functional characteristics of the mechanisms of particle removal by the three major cell-surface extensions. The data suggest that the underlying mechanisms, or at least the rate-determining steps, of the motile activities of lamellipodia, lobopodia, and filopodia are very similar, if not identical, although the morphological expressions of the mechanisms appear quite different.

DISCUSSION

Particle removal can proceed in the absence of extracellular nutrients or serum if certain minimal concentrations of salts are present in the extracellular fluid. Seemingly, these minimal conditions simply guarantee that the extracellular fluid maintains the correct osmolarity as well as a neutral or moderately alkaline pH. In view of the dependence of particle removal on Na+ and K+, one may also suspect that this composition of the extracellular fluid is necessary for the correct membrane potential. Therefore, one may assume that the motile activity of surface projections does not re-
Figure 7 Similarity of removal characteristics under the three conditions described in Fig. 6A, B, and C. (a) Temperature dependence at pH 7.8-8.0. (b) pH dependence at $T = 37^\circ C$. (c) Inhibition by cytochalasin B. The curves are designated as follows: salt, normal salt solution; salt + CS, normal salt solution with 10% calf serum added; and DME + CS, culture medium with 10% calf serum added. Abscissa in panel c indicates the concentration of cytochalasin B added to the various solutions. The curve designated as DMSO is the control in which the same amount of DMSO was added as was added together with the cytochalasin B from stock solutions.

The mechanism(s) of the motility of surface projections can be expressed predominantly in lamellipodia, in lobopodia, or in filopodia, depending on extracellular conditions that go beyond the minimal requirements. The presence of serum and the opportunity for anchorage to a solid substrate appear to be two major factors which determine the particular type of surface projection to perform extension-retraction cycles.

The surface movements which we have described in this paper precede and accompany the spreading of cells. We have previously (5) argued that filopodia and perhaps other surface protrusions as well probe the environment and induce cellular spreading upon firm attachment to a substrate. One would, therefore, expect similar dependencies on extracellular parameters of cell spreading and of surface projection movements. Indeed, the quantitation of macrophage spreading shows very similar results (24). However, the pH dependence of macrophage spreading is quite the opposite of the pH dependence of projection movement in 3T3 fibroblasts. This may suggest that the pH dependence of the movement of surface projections is dependent on the in vivo biological function of the respective cell line.

The microfilament organizations of filopodia, lamellipodia, and lobopodia are quite different (4, 6, 8, 10, 11, 29, 33). It is commonly believed that microfilaments are intimately involved in the motile mechanisms of nonmuscle cells. However, we found that particles can be removed by each of the three major surface projections despite their different microfilament organization. These results suggest that the surface projections have a motile mechanism in common which is independent of the microfilament organization and that the microfilament(s) do not require any specific signals from the fluid environment for its function.

Furthermore, the completion of extension-retraction cycles seems coupled to an active energy metabolism in the form of oxidative phosphorylation, or to glycolysis if the first pathway is inhibited. After trypsinization, the cells retain enough intracellular nutrients to maintain the motile activity of their surface protrusions for at least 1 h.
filament organization may be important only for the first two steps of an extension-retraction cycle, the extension and the attachment of surface projections (8, 21).

It seems to us that the third step of an extension-retraction cycle which physically removes the particles may be closely related to or identical with the well-known phenomenon of "centripetal surface flow" (3, 14, 18–20, 23). Indeed, Figs. 6A and B show that the removed particles flow centripetally along the surfaces of lamellipodia and lobopodia, and we have shown earlier that particles can also flow centripetally along stretched-out filopodia (6). It is easy to imagine that the second form of particle removal, namely complete retraction of a surface projections along with a particle (see Figs. 6 C), is observed when particle backflow and loss of attachment of the projections occur simultaneously. Therefore, the above described studies suggest to us the following possibility: the extension of various surface projections is caused by the association of microfilaments. Subsequently, the mechanism of the centripetal surface flow is expressed by the projections. This mechanism is intracellularly initiated and is coupled to the energy metabolism of the cells. It is dependent on pH and temperature and can be inhibited by cytochalasin B.

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