Cultured Megakaryocytes: Changes in the Cytoskeleton After ADP-induced Spreading

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ABSTRACT Megakaryocytes from guinea pig bone marrow were isolated and maintained in liquid culture and were treated with ADP, thrombin, arachidonic acid, or collagen. Megakaryocytes spread with an active ruffled membrane in response to ADP (1–100 μM), thrombin (1.0 U/ml), and arachidonic acid (50 μM) but responded to collagen surfaces only if fibronectin was added to the cultures. Spreading could be blocked completely by dibutyryl cyclic AMP (dibutyryl cAMP) or isobutylmethylxanthine at 1 mM, as well as by cytochalasin D (2 μg/ml), but not by colchicine up to 1 mg/ml.

The distribution of contractile proteins was examined by immunofluorescence. In untreated, spherical cells, staining with antimyosin, antifilamin, anti-α-actinin, or with fluorescein-labeled subfragment 1 (FITC-S1) was diffuse and unpatterned. With antitubulin antibody, however, microtubules were seen in a dense array throughout the unspread cells. In actively ruffling spreading cells, myosin, filamin, and actin were visualized in the region of the ruffled membrane while α-actinin was seen most prominently in a band located proximal to the inner part of the ruffle. In fully spread cells, actin, myosin, filamin, and α-actinin were seen in filaments that filled the cytoplasm. Antimyosin and anti-α-actinin staining of the filaments was periodic with ~1 μm center-to-center spacing. Actin, filamin, and α-actinin were also identified in punctate spots throughout the spread cytoplasm. Microtubules were absent from the ruffle but filled the cytoplasm of fully spread cells. Rings, 1.5–2.5 μm in diameter, were seen with antitubulin in 13% of the spread cells. Our results show that megakaryocytes respond to platelet agonists, but typically by spreading, rather than extending, filopodia. From the changes in localization of contractile proteins and from time-lapse cinematography, we propose a model for cell spreading.

Megakaryocytes are bone marrow cells of mammalian species that, by fragmentation of their cytoplasm, give rise to new blood platelets. Platelet formation requires both external and internal morphological changes in the megakaryocytes. As the megakaryocytes mature, they extend processes into venous sinusoids in the bone marrow which may be released as platelets (2, 19, 31). An internal structural change that must occur during platelet formation is the organization of microtubules into the characteristic circumferential coil seen in platelets (3, 29).

The presence of microfilaments and microtubules in megakaryocytes has been established by electron microscope studies (2, 4, 9, 34), but an overall picture of the distribution of these structures throughout the cell is lacking. Since indirect immunofluorescence is most useful for this purpose, we used this method to study the arrangement of the contractile and structural proteins of this interesting cell type. It had been reported earlier (11) that megakaryocytes in suspension could respond to platelet agonists. Using cultured megakaryocytes, we discovered a new response to platelet agonists. The cells on surfaces, when treated with several platelet-activating agents, form a typical ruffling membrane and spread out over the substratum. This spreading reaction is rapid, taking 20–30 min for completion, and reversible. We used this response to examine the distribution of contractile proteins in megakaryocytes during shape change and to study cell spreading in an easily controlled situation.

MATERIALS AND METHODS

Megakaryocyte Preparation

The preparative technique was developed by modification of two existing

methods (27, 32). All solutions used during the preparation were made in Levine's CATCH buffer (27), calcium- and magnesium-free Hanks' salt solution with 1.4 mM adenosine and 2.74 mM theophylline, pH 7.4. The humeri, femurs, and tibiae of two male Hartley guinea pigs (200–250 g) were removed and the adherent muscle and connective tissue were scraped off. The bones were cracked open and the marrow was scraped out and suspended by gentle pipetting in 15 ml of CATCH with 0.38% sodium citrate. The cell suspension was passed through nylon mesh (100-μm pore) and the cells were pelleted. The pellets were resuspended in 1 ml of CATCH with 0.38% sodium citrate and layered on top of a two-step bovine serum albumin gradient. The bottom step was 7.5 ml, refractive index 1.3697 (density 1.05 g/ml). The top step was 22.5 ml, refractive index 1.3630 (density 1.04 g/ml). The gradient was centrifuged at 10°C for 30 min at 10,000 *g* in a Beckman L5-50 centrifuge using a SW-27 rotor with 1 × 3.5-in. tubes. After centrifugation, the top step was removed and mixed with an equal volume of CATCH. The cells were pelleted and the pellet was washed in 10 ml of CATCH and repelleted. This pellet was resuspended in 1.0 ml of CATCH and layered on top of a 2%–4% continuous Ficoll gradient. The Ficoll gradient was 13 ml in a 17 × 100–mm test tube. The gradient was centrifuged at room temperature for 5 min at 100 *g*. The pellet from this gradient was the enriched megakaryocyte fraction.

Cultures

Isolated megakaryocytes were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 500 U/ml penicillin, and 50 μg/ml streptomycin. Cells were kept at 37°C in a 5% CO_2–95% air atmosphere. Cells were routinely cultured for 18–36 h before experiments.

Spreading

To compare populations objectively, cells were assigned to one of four classes: 0, spherical and unspread; +1, central raised area of the cell containing the nucleus wider than half the total cell diameter; +2, central raised area of the cell equal to half the total cell diameter; and +3, central raised area of the cell less than half the total cell diameter. At least 100 cells in a dish were assigned before and then, again, after the addition of ADP, and each class was expressed as a percent of the total population. The value for each class before addition of ADP was then subtracted from the value for each class after the addition of ADP so that the data could be expressed as the change of the percent of the total population in each class from before to after incubation with ADP (Δ% spread). This method allows each dish to serve as its own control and also corrects for a small number of cells that spread spontaneously. This was <1% of the cells and occurred only in occasional cultures.

FITC-S1

Fluorescein isothiocyanate–labeled heavy meromyosin subfragment 1 (FITC-S1) was prepared by modification of the method of Sanger (33). Chicken skeletal muscle myosin was prepared by the method of Huxley (18). Heavy meromyosin subfragment 1 (S1) was prepared by the method of Weeds and Taylor (41). Chicken skeletal muscle actin was prepared by the method of Spudich and Watt (35). S1 was dialysed against 0.1 M KCl, 50 mM Na_2CO_3 pH 8.5 for 3 h. F-actin in the same buffer was added in a 1:1 wt:wt ratio to the S1 (molar ratio actin to S1 of 2.5:1). FITC (3 mg/100 mg protein) and the S1-actin mixture were gently stirred for 3 h at 4°C. The actin-S1 complex was pelleted (2 h, 160,000 *g*, 4°C). The pellet was gently homogenized in standard salt (0.1 M KCl, 1 mM $MgCl_2$, 10 mM phosphate buffer, pH 7.0) with 10 mM dithiothreitol. The F-actin was pelleted again after adding 10 mM Mg-ATP to release the S1. The supernatant FITC-S1 was dialysed against standard salt overnight. The dialysed FITC-S1 was brought to 30% saturation with ammonium sulfate and the precipitate was discarded. The ammonium sulfate was brought to 50% saturation and the precipitate was pelleted and resuspended in standard salt. The FITC-S1 was then chromatographed on a 1.4 × 5.0-cm Sephadex G-25M column using standard salt, mixed with an equal volume of 100% glycerol, and stored at −20° C.

Fixation

Cells to be stained with antisera to myosin, α-actinin, filamin, or with FITC-S1 were washed three times with phosphate-buffered isotonic saline, pH 7.0 (PBS), at room temperature. Cells were then fixed in 3.8% formaldehyde in PBS for 10 min at room temperature followed by three washes with PBS. Cells were permeabilized in ice-cold 95% acetone for 7 min, then washed three times in PBS before staining. Cells stained with antiserum to tubulin were treated either by the above method or by the method of Osborn and Weber (30), except that GTP was not included in the solutions. In several preliminary experiments, we observed no differences in microtubule preservation whether or not GTP was present.

Staining

Antibody staining was carried out by applying enough antiserum diluted in PBS to coat a cover slip on which the cells were grown. Dilutions varied with different antisera but ranged from 1:30 to 1:50. The cover slip was inverted onto a petri dish and incubated at 37° C in a humid atmosphere for 1 h. The cover slip was rinsed three times in PBS and treated with the second antibody by the same procedure. The second antibodies were goat antirabbit immunoglobulin for myosin, α-actinin, and tubulin staining, and donkey antigoat immunoglobulin for filamin staining. The second antibodies were fluorescein conjugated. The bound fluorescein/protein ratios were 3.5 μg/mg for goat antirabbit and 4.25 μg/mg for donkey antigoat. After the second antibody the cover slip was washed three times with PBS and mounted in 90% glycerol–10% PBS with 3 mM NaN_3. FITC-S1 staining was done by first coating cover slips containing cells with FITC-S1 (1 mg/ml), inverting onto a petri dish on ice for 10 min, and then rinsing three times with cold PBS. The cover slips were then mounted as for antibody staining.

Photography

Cells stained for immunofluorescence were examined and photographed in an Olympus Vanox microscope or a Zeiss Photomicroscope II equipped with Zeiss planapochromatic objectives. Photographs were taken on Tri-X film and developed to yield an ASA of 1000.

Scanning Electron Microscopy

Megakaryocytes on cover slips were rinsed three times in Ca^{++}- and Mg^{++}-free Hanks' salt solution and fixed in 2% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, for 30 min at room temperature. After fixation, the cells were rinsed in the cacodylate buffer and postfixed in 1% OsO_4 in the same buffer for 1 h at 4°C. The cells were rinsed again in the buffer, then dehydrated with increasing concentrations of ethanol from 50%–100% for 10 min at each concentration, followed by three changes in fresh 100% ethanol. Cells were critical point–dried in a Denton DCP-1 dryer and coated with gold-palladium. Cells were examined and photographed in a Philips PSEM 500 scanner operated at 25 kV.

Transmission Electron Microscopy

Megakaryocytes grown on carbon-coated gold grids were lysed on the grid with 0.1 M KCl, 5 mM ethyleneglycol-*bis*(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA), 0.5% Triton X-100, 3 mM imidazole, pH 7.0 for 1 min. Cells were stained with 1% uranyl acetate and 0.02% cytochrome *c*. Samples were viewed in a Philips 201 electron microscope operated at 80 kV.

Time-lapse Cinematography

Cells on cover slips were inverted and sealed with paraffin over a well of medium. They were viewed with a 25× objective and filmed at 20 frames/min, using a Bolex camera on a Sage series 500 cinephotomicrographic apparatus.

Antibodies

The immune gamma globulin fraction from antiserum to myosin was a gift of Dr. J. R. Fallon (University of London), that to anti-α-actinin was a gift of Dr. K. Burridge (University of North Carolina), antiserum to tubulin was a gift of Dr. F. R. Frankel (University of North Carolina), and that to filamin was a gift of Dr. M. Willingham and Dr. I. Pastan (both of the National Institutes of Health). All the antisera have been previously characterized (7, 10, 37, 39).

Materials

Adenosine, theophylline, bovine serum albumin, ADP, thrombin, arachidonic acid, dibutyryl cAMP, and isobutylmethylxanthine were all obtained from Sigma Chemical Co. (St. Louis, Mo.). Goat antirabbit and donkey antigoat sera were purchased from N. L. Cappell Laboratories, Inc. (Cochranville, Pa.). Culture media, fetal calf serum, glutamine, and antibiotics were obtained from Gibco Laboratories (Grand Island Biological Co., Grand Island, N. Y.). Collagens and fibronectin were gifts of Dr. J. Lash (University of Pennsylvania). Taxol was a gift of Dr. H. Holtzer (University of Pennsylvania).

RESULTS

Megakaryocyte Preparation

The yield of megakaryocytes from a standard preparation as described was 100,000–400,000 megakaryocytes. The purity

ranged from 50% to 90%. If the Ficoll velocity gradient was repeated, the yield was at the low end of the range while the purity was at the high end of the range. The viability of cells tested by trypan blue exclusion was >90%. Megakaryocytes are easily distinguished from contaminating cell types because they are the only large (greater than ~20 μm in diameter), round cells in the bone marrow. These criteria have been shown to be reliable for recognizing megakaryocytes in bone marrow suspensions (26). We have cultured megakaryocytes on Falcon and Nuncware tissue culture plastic, types I, II, III, and IV collagen, poly-L-lysine, glass, or fibronectin. The cells remained round and were only slightly adherent to any of these substrates. With several vigorous rinses, most of the cells could be washed off the dish.

Response to Platelet-activating Agents

Cultured megakaryocytes were exposed to platelet-activating agents and observed by phase-contrast microscopy for morphological changes. We tested ADP (1–100 μM), arachidonic acid (50 μM), thrombin (0.1 and 1.0 U/ml), and types I, II, III, and IV collagen-coated surfaces. ADP at all concentrations tested, thrombin at 1.0 U/ml, and arachidonic acid all caused a dramatic spreading response. The normally spherical cells, 20–30 μm in diameter (Fig. 1 *a* and *c*), put out short filopodia, ~5 μm long and 0.7–1.5 μm in diameter, and became attached to the substrate. Then, in 5–10 min, the entire periphery of the cytoplasm began to ruffle as seen by time-lapse cinematography. The ruffling consisted of filopodia up to 10 μm long and

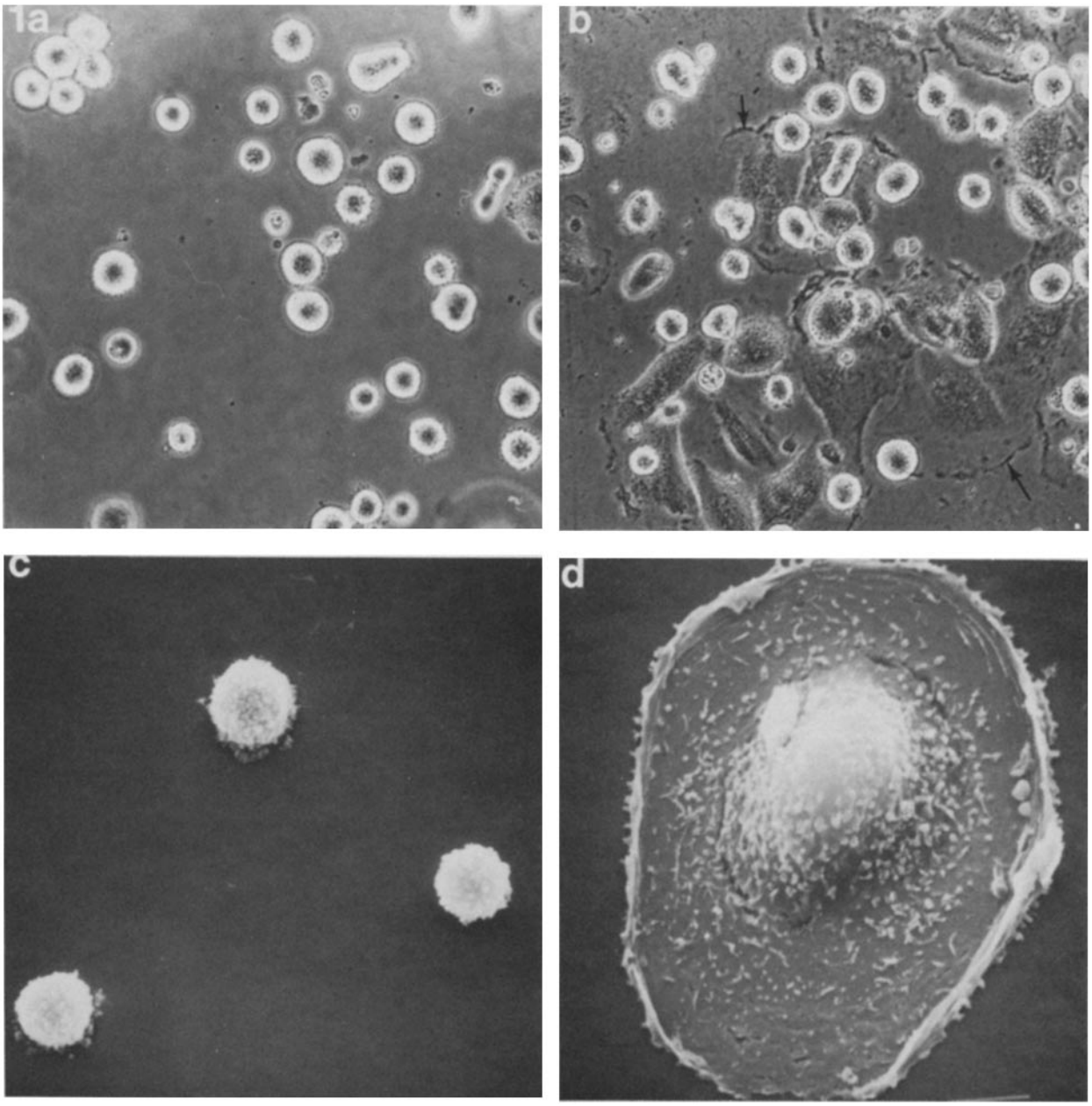

FIGURE 1 (*a*) Isolated megakaryocytes in liquid culture. Note the variation in size. × 240. (*b*) An identical culture after 30-min exposure to 10 μM ADP. Note the ruffled edges on the free margins of spreading cells (arrows). × 240. (*c*) Scanning electron micrograph of megakaryocyte culture. × 1,080. (*d*) Scanning electron micrograph of a cell that is almost fully spread; from culture in *b*. Note the thickness at the spreading margin. × 1,170.

1.5 μm in diameter projecting and receding and appearing to join lamellipodia. These lamellipodia continued to extend, while at the same time wavelike movements appeared to recede back toward the center of the cell. The ruffling region was a thickened arc 2–10 μm wide around the edge of the cell. As the ruffle moved out, it left a thin sheet of spread cytoplasm behind it (Fig. 1 *b* and *d*). By 35 min the cells were fully spread to a diameter of ~100 μm. The ruffle was gone, the cytoplasm was very thin and flat out to the edge of the cell, and the cells were now very adherent (Fig. 1 *b*, lower arrow). We previously reported that megakaryocytes did not respond to collagen type I (24). In this study we found that megakaryocytes did not respond to any of the types of collagen tested unless fibronectin (human cold-insoluble globulin) was added to the cultures on collagen surfaces at 5 μg/ml. Under these conditions, megakaryocytes on collagen surfaces spread in typical fashion. Twice, for unknown reasons, megakaryocytes responded to ADP by forming long filopodia 1 μm in diameter and 20–80 μm long instead of spreading (Fig. 2). These cells appeared similar to megakaryocytes observed in bone marrow smears after incubation in vitro at 37°C (36).

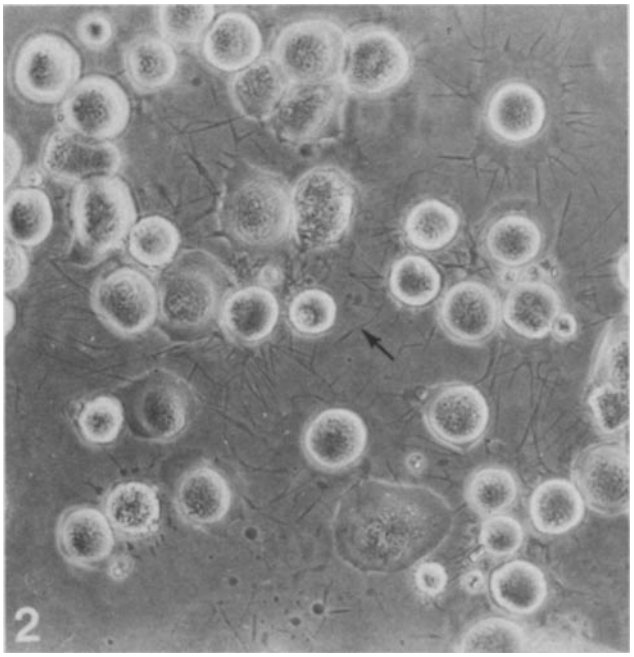

FIGURE 2 An unusual response to ADP. The cells have flattened and produced giant filopodia ~1 μm in diameter and 20–80 μm long. × 240.

All the results described below were obtained with megakaryocytes treated with 10 μM ADP, unless stated otherwise.

Characteristics of the ADP Response

The reaction to ADP was quite specific. It was not observed with uridine diphosphate, guanidine diphosphate, cytidine diphosphate, inosine diphosphate, or AMP, all at concentrations up to 50 μM. These compounds had no effect on megakaryocyte morphology. In most cell preparations ~30–50% of the megakaryocytes responded to ADP and spreading was complete in ~30 min (Fig. 3). Incubation of cells with 1 mM sodium azide and 1 mM sodium fluoride blocked spreading completely. Cells were treated with colchicine or Taxol to see whether microtubules were involved in ADP-induced spreading. Preincubation in colchicine (1 mg/ml for 30 min) did not inhibit spreading although it disassembled the microtubules as seen by indirect immunofluorescence. Taxol (10 μM) for 24 h did not affect cell spreading although it caused bundling of microtubules. On the other hand, preincubation in 2 μg/ml of cytochalasin D for 45 min blocked spreading completely. However, if cells were first stimulated to spread and then incubated with cytochalasin D, no morphological changes were observed. Even after a 6-h exposure to the drug, the cells maintained their spread configuration.

Although the spread cells appeared stable in cytochalasin D, they could be rapidly returned to the original rounded state by agents expected to increase internal cAMP levels. Dibutyryl

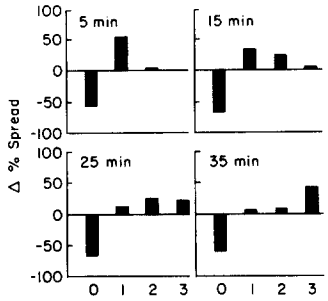

FIGURE 3 Time-course of ADP-induced spreading in a typical culture. Cells were assigned to size classes from 0 (spherical) to +3 (maximum spreading), as described in Materials and Methods, at times from 5 to 35 min after the addition of 10 μM ADP.

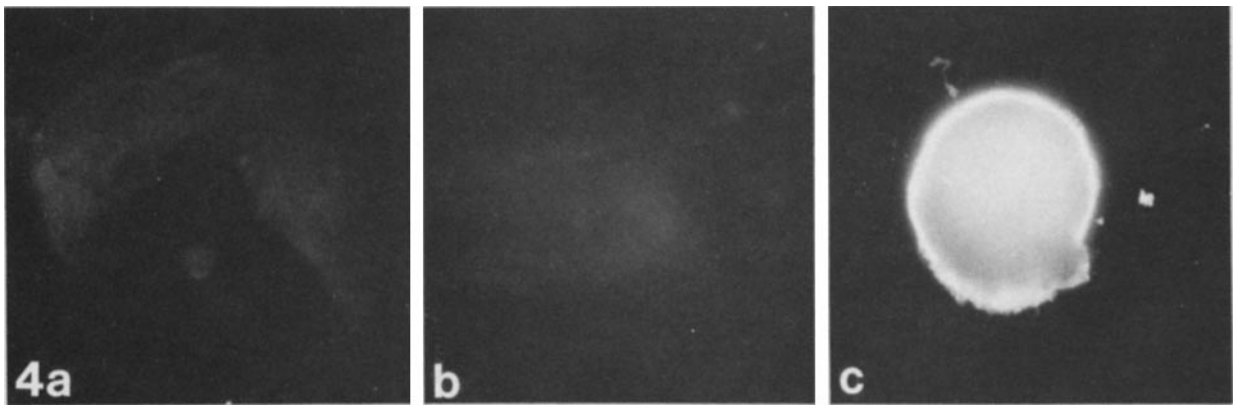

FIGURE 4 (*a*) Megakaryocyte culture stained with preimmune rabbit serum followed by FITC-goat antirabbit serum. × 700. (*b*) Megakaryocyte culture stained with FITC-goat anti-rabbit serum only. × 700. (*c*) Resting megakaryocyte in culture for 24 h, stained with antimyosin antiserum and FITC goat antirabbit serum. × 700.

cAMP (1 mM) and isobutylmethylxanthine (1 mM) separately or together reversed spreading completely in 30–45 min. Cells preincubated in the drugs were also completely blocked from spreading when stimulated by ADP, thrombin, or arachidonic acid. Cells that have been spread with ADP and rounded up by incubation with dibutyryl cAMP and isobutylmethylxanthine can be respread by washing out the drugs and exposing the cells to ADP again. This cycling has been used to enrich for a population of ADP-responsive cells. We also observed that 1.5 mM tetracaine inhibited and reversed spreading.

Immunofluorescence

To study the distribution of cytoskeletal structures in megakaryocytes, we used indirect immunofluorescence with anti-

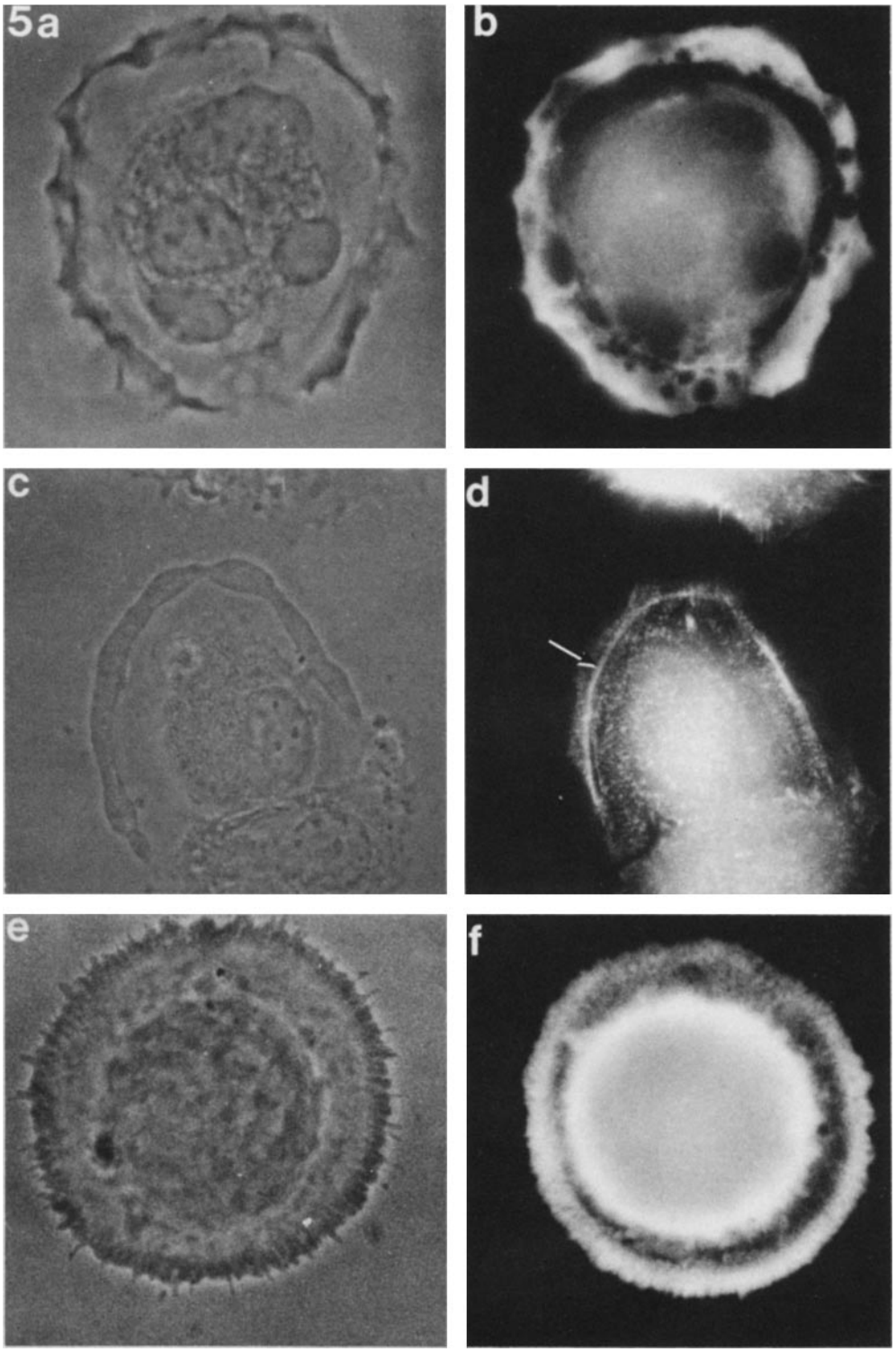

FIGURE 5 Representative views of megakaryocytes exposed to 10 μM ADP for 10 min. *a, c,* and *e* are phase-contrast microscope views of *b, d,* and *f*. Note that *f* is an early stage with short filopodia projecting from the developing ruffle. (*b*) Stained with antimyosin antiserum as described in Materials and Methods. × 1,600. (*d*) Stained with anti-α-actinin antiserum. × 630. (*f*) Stained with antifilamin antiserum. × 1,600. Note the bright staining of the ruffle in *b* and *f* and the prominent band of staining behind the ruffle in *d* marked by the arrow.

bodies to myosin, α-actinin, filamin, and tubulin and FITC-S1 to localize actin. Controls showed no staining with preimmune rabbit serum followed by FITC-goat antirabbit (Fig. 4*a*) or with the labeled goat antirabbit alone (Fig. 4*b*).

Myosin

The distribution of myosin in unspread cells was diffuse, unpatterned, and relatively homogeneous throughout the cytoplasm (Fig. 4*c*). This was true also for the distribution of filamin, α-actinin, and actin (not shown). After the megakaryocytes were stimulated with ADP and began to spread and ruffle, the staining with antimyosin remained diffuse but was very strong in the ruffle (Fig. 5*a* and *b*). As the cells continued to spread, filamentous structures that stained with a periodicity of ~0.9–1.0 μm became visible in the flattened cytoplasm behind the ruffle. When the cells were fully spread, the flattened cytoplasm was filled with these filaments. These structures were found oriented circumferentially around the nucleus in the center of the cells (Fig. 6*a*) or, in other cells, in straight bundles running like chords of a circle (similar to the actin distribution shown in Fig. 7*d*) roughly perpendicular to radii of cells.

α-Actinin

The distribution of α-actinin in ADP-treated cells was quite different from that of myosin. As the cells began to spread and ruffle, punctate staining appeared throughout much of the cytoplasm and there was only very weak staining of the ruffle. A band appeared just proximal to the ruffle that stained intensely with antisera to α-actinin (Fig. 5*c* and *d*). As the cells became more fully spread, α-actinin antibody showed circumferential filamentous staining that was periodic and radial filamentous staining that was not periodic. Also, punctate spots of anti-α-actinin staining about 0.8–1.1 μm in diameter were seen distributed throughout the cytoplasm (Fig. 6*b*).

Filamin

In spreading cells the staining for filamin was intense in the ruffle (Fig. 5*e* and *f*). When the cells were fully spread the cytoplasm was filled with circumferential filaments. The staining along these structures was continuous. Punctate staining could also be seen throughout the spread regions of the cell (Fig. 6*c*). These spots often seemed to be lying along the fibrous structures. As with myosin, the filaments were circumferential or in parallel sheets running perpendicular to the radius of the circular cells.

FITC-S1

The specificity of the FITC-S1 staining was tested in a system where the appearance of actin-containing structures has been well characterized (20, 21, 33). Chick embryo fibroblasts were stained with FITC-S1 in the presence and absence of 10 mM MgATP. In the absence of MgATP, fibroblasts showed typical stress fiber staining (Fig. 7*a*). If the staining is specific for actin, then the staining should be blocked in the presence of MgATP. In fact, the staining was almost completely eliminated if 10 mM MgATP was present during staining (Fig. 7*b*). In megakaryocytes that were beginning to spread and ruffle in response to ADP, strong staining was seen in the ruffle (Fig. 7*c*). Fully spread cells showed a pattern of staining essentially identical to that seen with antifilamin staining. Circumferential fibrous structures with no periodicities filled the spread cytoplasm. As with antifilamin and anti-α-actinin staining, FITC-S1 also stained punctate spots that were found throughout the spread cytoplasm (Fig. 7*d*). Note the difference in final ori-

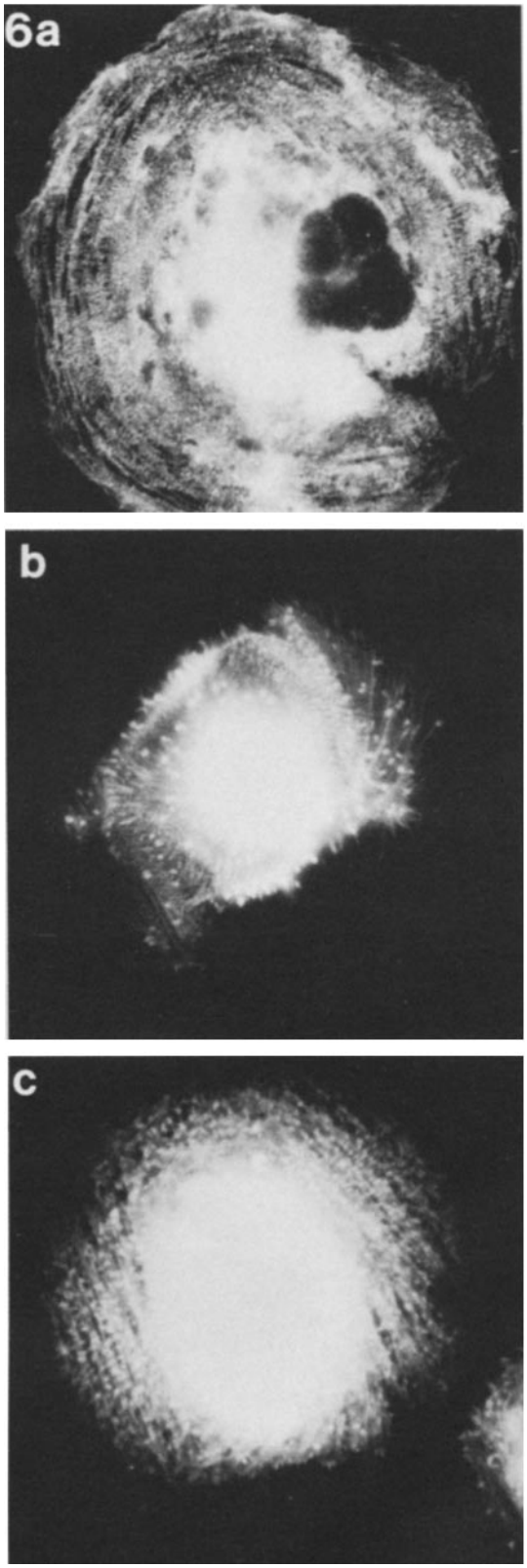

FIGURE 6 More fully spread megakaryocytes after exposure to 10 μM ADP for 30 min. (*a*) Stained with antimyosin antiserum. × 813. (*b*) Stained with anti-α-actinin antiserum. × 630. (*c*) Stained with antifilamin antiserum. See text for discussion. × 813.

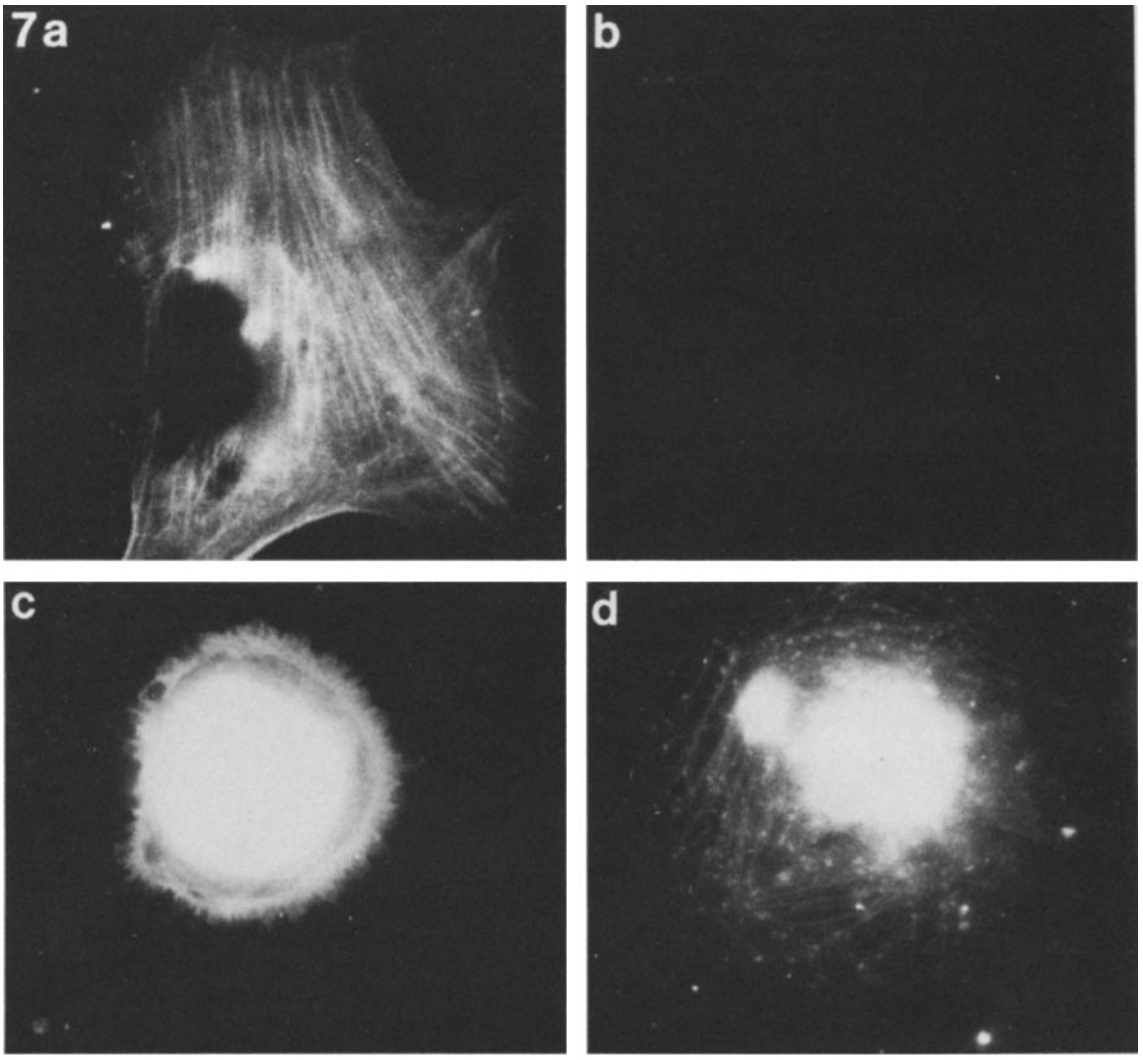

FIGURE 7 (*a*) Chick embryo fibroblast stained with FITC-S1. × 975. (*b*) Chick embryo fibroblasts stained with FITC-S1 in the presence of 10 mM Mg-ATP. Cells were photographed and negatives printed with the same treatment as in *a*. × 975. (*c*) Megakaryocyte exposed to 10 μM ADP for 10 min and stained with FITC-S1. × 975. (*d*) Megakaryocyte exposed to 10 μM ADP for 30 min and stained with FITC-S1. × 975. Even in this well-spread megakaryocyte the center of the cell remains thick (see Fig. 1 *d*) and stains heavily.

entation of filament bundles in a fibroblast, where they are elongated perpendicular to the cell margin, and in the megakaryocyte where the bundles lie parallel to the cell margin.

Tubulin

The distribution of microtubules in these cells is of particular interest because during maturation of megakaryocytes they give rise to the microtubule coil in platelets. When spherical, unstimulated megakaryocytes were stained with antiserum to tubulin, an extensive array of convoluted microtubules was seen throughout the cytoplasm (Fig. 8 *a*). After megakaryocytes were exposed to ADP and spread, the microtubules were found throughout the cytoplasm but were not seen in the ruffle. A very striking feature in 13% of the spread cells was the appearance of ring structures (Fig. 8 *b*, arrows). The rings were 1.5–2.5 μm in diameter and from 10 to 25 were seen per cell. They were found in all areas of the cytoplasm. We used colchicine and taxol to see whether differential loss or stabilization of the rings could be produced, but no difference in the reactivity of the rings as compared to the other microtubules was found.

Electron Microscopy

Scanning electron micrographs of megakaryocytes confirmed at a finer level what was seen by phase-constant microscopy. The spreading cells first put out short filopodia, then developed a thickened ruffle (Fig. 1 *D*), spread, and finally flattened. By transmission electron microscopy, two prominent features seen in the immunofluorescence procedure were also present. The cytoplasm of spread cells was filled with filament bundles whose distribution appeared the same as that of those stained with antisera to myosin, filamin, and α-actinin and FITC-S1. Electron-dense bodies 0.15–0.35 μm in diameter were seen throughout the cytoplasm (Fig. 9). Their distribution appeared similar to punctate staining seen with antifilamin, anti-α-actinin and FITC-S1. Compare Fig. 9 with Fig. 6 *c*.

DISCUSSION

Only recently have adequate methods been developed for the isolation of relatively pure populations of megakaryocytes (27, 32). Consequently, the physiology of this remarkable cell is

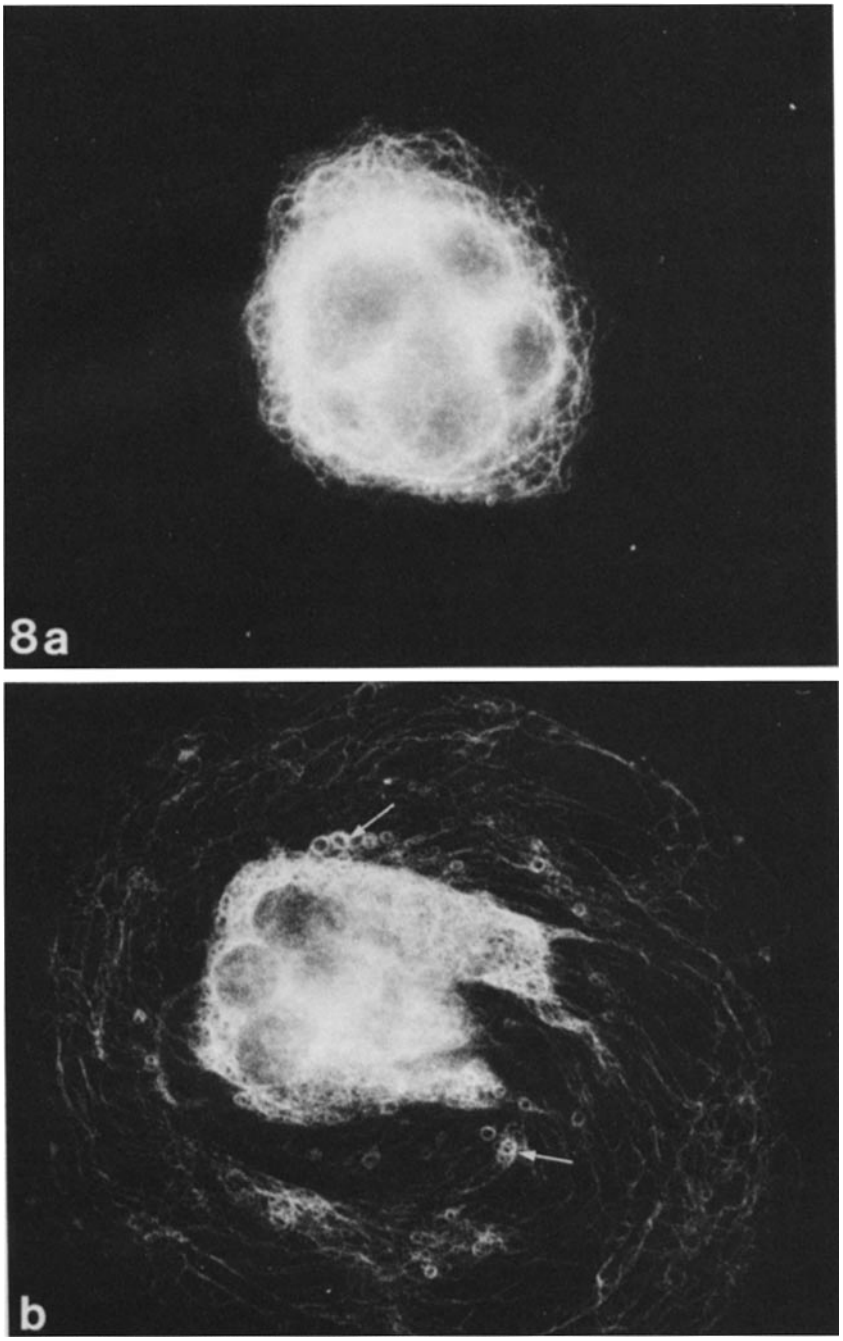

FIGURE 8 (*a*) Resting megakaryocytes stained with antitubulin antiserum. × 1,430. (*b*) Similar culture exposed to 10 μM ADP for 30 min and stained with antitubulin antiserum. × 1,430. Note the ring-shaped structures at different sites within the cell (see arrows). These measure 1.5–2.5 μm in diameter.

only beginning to be understood (11, 12, 13, 25, 28). In particular, it is not known what characteristics the megakaryocytes share with their progeny, platelets. We found that the platelet agonists ADP, thrombin, arachidonic acid, and collagen together with fibronectin all caused cultured megakaryocytes to ruffle and spread. Yet, at best, only ~50% of the cells spread. Why the other 50% of the cells do not is unknown. Since 90% of the cells exclude trypan blue, it is not a question of viability. It may be that the cells that do not spread are less mature and have not developed the ability to respond to these agents, which probably requires surface receptors. In support of this conclusion we found that >90% of the cells spread when treated with the ionophore A-23187 and methylamine together (23). Spreading induced by ADP, thrombin, and arachidonic acid was inhibited by inhibitors of platelet activation, dibutyryl cAMP, and isobutylmethylxanthine. The effect of inhibitors on collagen-induced spreading was not tested. Despite the great difference in cell size, megakaryocyte spreading shares two features with spontaneous platelet spreading. Platelets activated by a glass surface initially put out filopodia which adhere, and then the intervening spaces are filled in by an advancing sheet of cytoplasm (1). Megakaryocytes also put out filopodia initially and at the same time begin to adhere. Platelet filopodia are ~2 μm in length, and spreading continues out to the end of these filopodia. In megakaryocytes the filopodia are ~5 μm long, and spreading continues well past the ends of the filo-

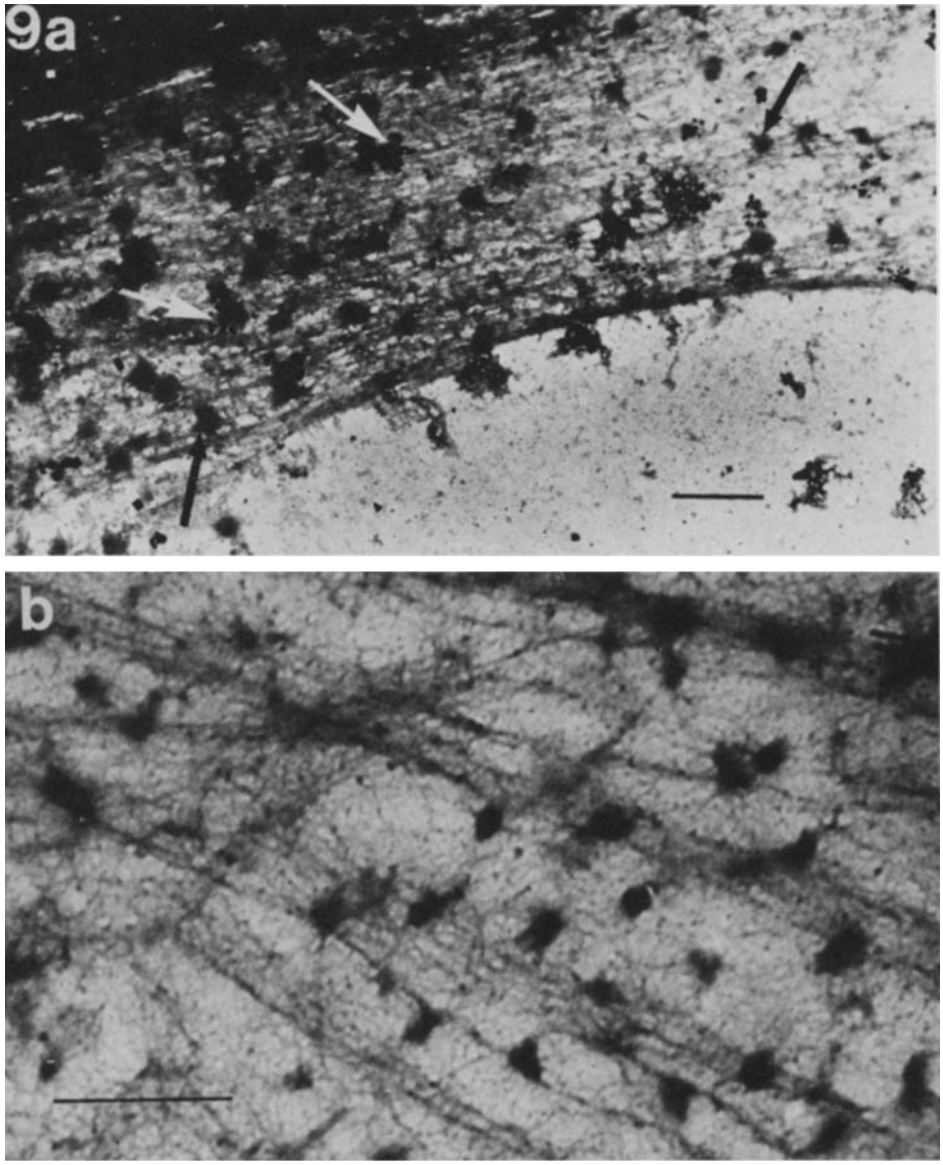

FIGURE 9 Megakaryocytes cultured on gold grids and treated with 10 μM ADP for 30 min. The cells were lysed and stained as described in Materials and Methods. (*a*) Filamentous material as well as densities (black arrows), similar in distribution to spots staining with antifilamin, anti-α-actinin, and FITC-S1, are seen. This area shows stain crystals (white arrows) to illustrate how the densities and stain crystals can be easily distinguished from each other. *a*: bar, 2 μm. × 6,400. *b*: bar, 1 μm. × 24,000. The densities are about 150–350 nm in diameter.

podia. The presence of an active ruffle is an easily observed feature of megakaryocyte spreading, but a small ruffle has been seen around the periphery of platelets observed by surface reflectance microscopy (R. D. Allen, personal communication). These results show that there are strong resemblances between the giant precursor cell and the fragment of cytoplasm it produces, and, therefore, the study of megakaryocyte spreading is valuable for complementing studies on platelet activation as well as for learning about megakaryocytes themselves.

The requirement for fibronectin in the collagen response could be that fibronectin somehow links the megakaryocyte and the collagen substratum allowing interactions for spreading. During the cell preparation the megakaryocytes are washed many times and may lose fibronectin normally associated with the cell surface. This may be why additional fibronectin must be added back to cultures to get a response to collagen. It should be noted that fibronectin alone causes no spreading. It has been reported, however, that fibronectin is required for fibroblast spreading only on dried collagen surfaces (which we used here) but not for spreading on native collagen gels which we have not used (17).

Microtubules have been identified by several workers in megakaryocytes by electron microscopy but no unusual arrangements of microtubules have been observed. We were therefore surprised to find microtubule rings in some of the ADP-treated megakaryocytes. From our observations we cannot conclude whether the rings existed before spreading and only became visible after spreading, or whether the rings actually formed during spreading. The microtubules are so densely packed in the unspread cells that the rings could not be resolved if they existed before spreading. We have tried, so far unsuccessfully, to answer this question by compressing unspread cells. Since rings have not been seen by electron microscopy of cells in the marrow, it is possible that rings do

not form until after stimulation by ADP. If so, there is not a good correlation between maturity as determined by the ability to spread and maturity as determined by the presence of rings, since only 13% of the cells possess the rings. Possibly, the cells with rings represent the most developed group of megakaryocytes. In any case, since the rings are remarkably similar in size to platelets (1.5–2.5 μm in diameter), it is attractive to speculate that the rings represent forming platelets.

Microtubules have been seen in pseudopodial extensions of megakaryocytes in bone marrow, and it has been suggested that they play a role in forming the pseudopodia (2). In our observations on both filopodial formation and ruffling, we have seen no evidence for involvement of microtubules. Colchicine does not affect any of the movements and we do not see microtubules by immunofluorescence in filopodia or ruffles.

The response of megakaryocytes to ADP presents a good opportunity to study cell spreading. It is relatively fast and reversible. Morphologically, the early stages of megakaryocyte and fibroblast spreading appear very similar (38, 42). First observed is the projection of short filopodia, followed by movement of a sheet of cytoplasm out in a radial fashion with a ruffling edge. Later, after the initial stages of spreading are complete, fibroblasts become polygonal and begin to migrate. Megakaryocytes remain circular or become only slightly polygonal and do not move about. The initial stages of megakaryocyte spreading, however, like those of fibroblast spreading, are not affected by colchicine or colcemid (15, 38). Therefore, findings from the study of megakaryocyte spreading may be applicable more generally to cell spreading.

Treatment of unspread megakaryocytes with antisera to myosin, filamin, and α-actinin and FITC-S1 gave the same results: diffuse, unpatterned staining. This result would be expected if the proteins are nonfilamentous and evenly distributed throughout the cytoplasm of the rounded cells. It is also possible that in unspread cells these proteins are in filamentous structures so densely packed together that individual filaments are not discernible. It should be noted that distinct microtubules can be clearly seen in rounded cells, though, so that close packing of filaments seems less likely. A definitive answer will require more detailed ultrastructural study.

When the cells are actively spreading, the ruffled margin contains actin, myosin, and filamin but virtually no detectable α-actinin or tubulin. The α-actinin is seen in a prominent band just behind the ruffle. No geodesic dome structures, a suggested intermediate in cell spreading (20), have been seen in hundreds of cells observed. The fully spread cells contain filaments throughout their cytoplasm. These are most likely actin microfilaments with myosin, filamin, and α-actinin bound to the actin. We have not been able to detect phase-dense stress fibers in living cells, but the immunofluorescent staining of megakaryocyte filaments is similar to the staining of stress fibers described in other cells (21, 22, 40). In addition to stress fibers, we observed a distinct punctate staining with antifilamin, anti-α-actinin and FITC-S1 but not with antimyosin. We found similar densities in the whole-mounts viewed by electron microscopy. We doubt that these represent collapsed microvilli. Microvilli seem, by scanning electron microscopy, no more numerous on these cells than on others, yet this staining pattern has not been previously reported for other cell types, nor do we see these densities in our own observations of fibroblasts. It is interesting to note that these same three proteins, filamin (or actin binding protein), actin, and α-actinin, are found in cytoskeletal precipitates of resting platelets that appear granular (16). We speculate that the treatment of megakaryocytes with platelet agonists causes cytoskeletal reorganization but leaves unaltered or even aggregates the proteins in some regions involved in platelet maturation.

Somewhat similar findings have been observed in a study of spreading granulocytes (5), which noted circular densities associated with a filamentous net on the cytoplasmic face of plasma membranes of these cells. The densities were reported to be 150–200 nm in diameter by scanning electron microscopy and 4–7 nm by transmission electron microscopy. The punctate structures we have seen in megakaryocytes using immunofluorescence are ~1 μm in diameter and by transmission electron microscopy the densities with identical distribution are 150–350 nm in diameter. There is considerable difference in the size of the densities in spreading granulocytes and megakaryocytes, although in both cases the densities are associated with filamentous material. By using the immunofluorescence method, it should be possible to determine whether the densities in spreading granulocytes also contain α-actinin, filamin, and actin, as in megakaryocytes. A second similarity between our results and those of Boyles and Bainton (5) is the appearance of circumferential bundles of filaments in spreading cells, indicating that this may be a common formation during spreading before cells have polarized.

Our results suggest a model for cell spreading. An appropriate stimulus (here ADP) at the cell surface triggers changes in the cell that cause first the protrusion of filopodia. From all that has been observed in different types of cells, this is likely to occur by the polymerization of actin into filament bundles. The polymerization pushes out a process surrounded by the cell membrane. Then the area between the filopodia begins to polymerize, but now into nets of microfilaments, and these fill in the gaps. The cytoplasm begins to ruffle, perhaps involving local gel solutions and contractions as proposed by Condeelis and Taylor (8), and the ruffle pushes out the border of the cell by this rather different process which involves the contractile activity of actin and myosin. We hypothesize that the band of α-actinin seen next to the ruffle represents an anchoring region. Contraction would then cause outward movement of the free margin of the cell because the proximal edge is held in place on the substratum by the connection to the α-actinin. As the margin moves outwards, filamentous structures continue to form in the spreading cytoplasm and become aligned predominantly parallel to the edge of the cell, as we see them in immunofluorescence, suggesting that the α-actinin band may function to align the filaments as they form.

This model is admittedly speculative, but it does take into account all of our observations: the presence of actin and myosin in the ruffle, the positioning of α-actinin, and the alignment of the final filaments. The inhibition of spreading by cytochalasin D implies that actin polymerization is required for spreading (6, 14) and probably for the formation of the filaments seen in the spread cells. Hence, these filaments may provide the structural rigidity to maintain the cell in the extended configuration. Since megakaryocyte spreading is not spontaneous and since these cells do not polarize and migrate, it remains to be seen whether this model describes spreading by other cell types.

How ADP causes these changes is an intriguing question. Our preliminary experiments (23) in which the ionic environment is varied and different ionophores and methylamine are used to stimulate megakaryocytes suggest roles for Ca^{++}, Na^{+}, and H^{+} ion changes in the process which triggers spreading.

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Note Added in Proof: Dr. Craig Thompson (National Naval Medical Center) has measured the sizes of microtubule rings in several spread megakaryocytes stained with antitubulin from our preparations and found the size distribution of the rings is the same as the size distribution from a normal population.

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