Interactions of Cytoplasmic Granules with Microtubules in Human Neutrophils

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Abstract. Ultrastructural and functional studies of degranulation responses by human neutrophils have suggested that microtubules (MTs) have a role in the intracellular transport of neutrophil granules. We have found that granule–MT complexes can be isolated from disrupted taxol-treated (1.0 μM) neutrophils, visualized by electron microscopy, and quantified in terms of granules per MT length. After incubation of neutrophils with the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), granule–MT complex formation was found to be increased two- to threefold. Enhanced binding of granules to MTs was detectable within 30 s of fMLP stimulation and was dependent on the concentration of fMLP. Incubation of cells with dibutyryl cAMP inhibited this fMLP-stimulated granule–MT complex formation in a dose-responsive fashion. These granule–MT interactions could be reproduced in a cell-free system with neutrophil granules isolated by density gradient centrifugation and MTs polymerized from phosphocellulose-purified tubulin. Furthermore, reconstituted granule–MT interactions were found to be modulated by ATPase inhibitors. Sodium orthovanadate increased granule–MT interactions in a concentration-dependent manner, while AMP-PNP, a nonhydrolyzable ATP analogue, and *N*-ethylmaleimide decreased or eliminated these interactions. In addition, we found that a MT-activated ATPase could be recovered from intact neutrophil granules by salt extraction, and that extracts enriched in this ATPase contained a polypeptide of between 115 and 120 kD which binds ATP and is immunologically related to kinesin. These studies demonstrate that cytoplasmic granules interact with MTs in human neutrophils in a regulated stimulus-responsive manner, and they suggest that such interactions may involve an MT-based, ATPase-dependent, vesicle translocation system as has been demonstrated in other types of cells.

GRANULE mobilization and degranulation are important components of neutrophil function in inflammation and host defense against microbial infection (15, 53). Yet, the basic mechanisms by which cytoplasmic granules are translocated to the plasma membrane and to phagosomes in activated neutrophils before membrane fusion are not clearly understood. In the early 1950's, Bessis and coworkers (4, 35) reported microcinematographic studies of living, spread human neutrophils in which they observed that granule movements are not entirely random but follow linear paths that radiate from the center of the cell. They concluded that cytoskeletal structures organize and orient such granule movements in neutrophils. Subsequent studies by others have indicated that these cytoskeletal structures are likely to be microtubules (MTs)[1].

MTs have been shown to organize and facilitate the movement of cytoplasmic organelles in a variety of different cell types (2, 14, 21, 28, 43, 46). Furthermore, it has been shown that dibutyryl cGMP, or agents which raise cellular cGMP levels (e.g., cholinergic agonists), increase the formation of MTs in neutrophils and enhance degranulation responses (12, 18, 52, 57). On the other hand, dibutyryl cAMP and agents which raise cAMP levels (e.g., theophylline, prostaglandin E_1, or β-adrenergic agonists) have been shown to have opposite effects (19, 52, 57) and also to inhibit the alignment of granules with MTs which may be observed in thin-section electron micrographs of neutrophils (52). A role for MTs in the mobilization of neutrophil granules has also been suggested by the effects of MT-disrupting agents on these cells. Colchicine and vinblastine have been shown to suppress both intracellular and extracellular degranulation responses in neutrophils under conditions in which these drugs prevent tubulin polymerization and MT formation (24, 25, 55, 58).

In the studies reported here, we have explored the possibility that ATPase-mediated interactions between MTs and storage organelles occur in human neutrophils as in other types of cells (e.g., neuronal cells [22, 33, 43, 49], chromatophores [27], and pancreatic islet cells [10]) and may have a role in the intracellular organization and transport of neutrophil granules. We show that granule–MT complexes form in intact neutrophils and can be recovered after disruption of

1. *Abbreviations used in this paper*: AMP-PNP, 5′-adenylimidodiphosphate; DIC, differential-interference contrast; fMLP, *N*-formyl-methyl-leucyl-phenylalanine; G/MT, granules per microtubule length; MT, microtubule; PC, phosphocellulose; PEM buffer, Pipes/$MgSO_4$/EGTA buffer.

the cells. Furthermore, the formation of these granule–MT complexes is shown to be a regulated event that is stimulated during neutrophil activation. We also demonstrate that granule–MT complex formation can be reproduced in an in vitro, cell-free system and modulated by ATPase inhibitors. Finally, we show that a MT-activated ATPase activity is associated with and may be recovered from isolated, intact neutrophil granules.

Materials and Methods

Isolation of Human Neutrophils

Neutrophils were isolated from the venous blood of healthy volunteers by centrifugation through Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ), followed by dextran sedimentation and the removal of residual red blood cells by hypotonic lysis as described previously (6, 54). These neutrophil isolates (>96% pure) were resuspended in Hanks' balanced salt solution without calcium or magnesium. For certain studies, large numbers of neutrophils ($\geq 5 \times 10^9$) were isolated from leukocyte concentrates obtained from normal volunteers by continuous flow centrifugation leukapheresis.

Video Microscopy

Video-enhanced, differential-interference contrast (DIC) microscopy was used to examine granule movements in intact, living neutrophils and to monitor granule–MT complex formation in cell-free systems. For the former studies, unactivated neutrophils were resuspended in Hanks' balanced salt solution and allowed to spread on glass slides before videotaping under phase-contrast or DIC optics. For studies of granule–MT complex formation, isolated granules were suspended in cavitation buffer and mixed with either *Chlamydomonas* axonemes or taxol-stabilized MTs polymerized from phosphocellulose (PC)-purified rat brain or chicken erythrocyte tubulin. After a 5-min incubation at 37°C the samples were viewed in an Orthoplan microscope (E. Leitz Inc., Rockleigh, NJ) equipped with a 100×/1.32 numerical aperture NPL/Fluotar ICT objective and a 12-V, 100-W tungsten halogen lamp. Images were projected to a Hitachi closed-circuit TV camera (Hitachi-Denshi, Ltd., Tokyo, Japan) and then further processed by an image processor (model DS-580; Quantex, Sunnyvale, CA). The video-enhanced images were recorded in real time with a JVC CR 6060U 3/4-inch video cassette recorder.

Isolation of Granule–MT Complexes

Neutrophils were suspended in a Pipes buffer (10 mM Pipes, pH 6.9, 100 mM KCl, 3 mM NaCl, 1 mM PMSF, 0.1 mM leupeptin, 0.1 μg/ml aprotinin [45]) containing 1 mM ATP. Taxol (a gift of Dr. Matthew Suffness, National Cancer Institute, Bethesda, MD) was added (1.0 μM, final concentration) for a 20-min incubation at 37°C to stabilize MTs. The cells were either sonicated three times for 10 s or subjected to 400 psi nitrogen at 4°C in a Kontes minibomb (Kontes Glass Co., Vineland, NJ) to disrupt the cells by nitrogen cavitation (5). Cell sonicates or cavitates were then centrifuged at 100 *g* for 5 min to remove residual intact cells and nuclei. Samples were applied to carbon-coated parlodian electron microscope grids either with or without fixation in 1% glutaraldehyde. For certain studies, cells were incubated with various concentrations of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) (Sigma Chemical Co., St. Louis, MO) from 30 s to 10 min with and without dibutyryl cAMP (10^{-8}–10^{-5} M) (Sigma Chemical Co.) before taxol treatment and disruption. Samples were then applied to electron microscope grids without centrifugation or fixation and examined by EM.

Separation of Neutrophil Granules

100–200 million neutrophils, suspended in 5 ml Pipes buffer, pH 6.9 (see above), containing 20 μM ATP, were disrupted by nitrogen cavitation and collected into Pipes buffer supplemented with EGTA (final concentration, 1.25 mM). After cavitation, the disrupted cell suspension was centrifuged at 230 *g* for 10 min to remove unbroken cells and nuclei. The granule-rich supernatant was then layered onto a 30-ml discontinuous sucrose gradient (steps of 30, 45, and 60% sucrose) and centrifuged at 100,000 *g* for 7 h. The cell cavitate separated into three organelle-rich bands: a band of membrane vesicle fragments at the sample/sucrose interface; a low density granule-rich band at the 30/45% sucrose interface enriched with peroxidase-negative, secondary granules; and a higher density band at the 45/60% sucrose interface containing peroxidase-positive, primary granules. This method achieved a separation of plasma membrane vesicles, secondary granules, and primary granules similar to that achieved with discontinuous Percoll gradients (6); it did not, however, separate the two distinct primary granule populations that can be resolved with continuous sucrose gradients (56).

Preparation of MT Protein and MTs

Chicken, cow, or rat brain tubulin was isolated by the method of Dentler et al. (11) in 0.1 M Pipes buffer containing 1 mM $MgCl_2$, 2 mM EGTA, 1 mM GTP, and 4 M glycerol. Chicken erythrocyte tubulin was prepared from chicken blood by the method of Murphy and Wallis (29). Tubulin was purified free of MT-associated proteins by ion-exchange chromatography using Whatman Inc. (Clifton, NJ) P-11 PC (39) and processed through a cycle of MT assembly and disassembly before use to remove any inactive subunits. Unless stated otherwise, MT assembly buffer was 0.1 M Na-Pipes, pH 6.94, containing 1 mM $MgCl_2$, 1 mM GTP, and supplemented with 5% glycerol. Purified tubulin was concentrated by polymerizing PC-tubulin in 0.1 M Pipes, pH 6.94, containing 10 mM $MgCl_2$, 1 mM GTP, and 20% glycerol, sedimenting, and resuspending at 2–3 mg/ml in assembly buffer as described above. MTs were then polymerized from this protein preparation and stabilized with 1–5 μM taxol for granule interaction studies.

Isolated protozoal axonemes were prepared from *Chlamydomonas reinhardtii* by the method of Allen and Borisy (1). These organisms were washed twice with distilled water followed by a wash with 10 mM Hepes buffer, pH 7.15, containing 0.5 mM β-mercaptoethanol, 0.5 mM EDTA, and 5.0 mM $MgCl_2$. The algae were then deflagellated by the addition of NP-40 to a final concentration of 0.4%. Gentle pipetting of the solution produced demembranated axonemes with characteristic splayed distal ends, allowing for a determination of structural polarity. Axonemes were separated from the cell bodies by two centrifugations at 350 *g*, dispensed into 200-μl aliquots, and frozen at −80°C.

Preparation of Granule–MT Complexes in a Cell-free Reconstituted System

Granules were prepared from isolated human neutrophils as described above and used either as mixed granules or further separated into heavy and light density (primary and secondary) granule fractions. The granules were diluted to 0.1–0.2 mg/ml protein content with 100 mM Pipes, pH 6.94, containing 1 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. 100-μl aliquots of the granules were then mixed with 10 μl taxol-stabilized (10 μM) chicken brain MTs at a final tubulin concentration of 0.05–0.15 mg/ml. Typically, the concentrations of reconstituted MTs and granules in these experiments were in the range of 0.05 mg/ml tubulin and 0.1–0.3 mg/ml granule protein, respectively. These concentrations were chosen in order to approximate the ratio of granules to MTs in intact neutrophils. Assuming an average granule diameter of 0.5 μm, an average cell volume of 300 μm^3, and one third of the cell volume occupied by granules, one can estimate that the average cell might have 1,000–2,000 granules. Studies of MTs in neutrophils have suggested that these cells have ~30–40 MTs of 4–5 μm in length. These estimates would suggest granule–MT ratios in the intact cell of 6–7 granules/μm of MT length or 25 granules/MT. In our reconstituted system, there were $\sim 4 \times 10^9$ granules (2×10^7 granules/μl) and 5×10^5 MTs ~3-μm long (based on 1,675 dimers/μm of MT length) for a ratio of 8 granules/MT. Similar experimental conditions were used to study the effects of various modulators of ATPase activity. ATP, sodium orthovanadate, 5′-adenylimidodiphosphate (AMP-PNP), and *N*-ethylmaleimide were added from concentrated stocks to give the desired concentrations. Reaction mixtures were then incubated at 37°C for 5 min before samples were applied to electron microscope grids, negatively stained with uranyl acetate, and examined for granule–MT complexes. Since the absolute numbers of granules varied in each of the experiments using the reconstituted system, the effects of ATPase inhibitors were always evaluated against control conditions run with each experiment.

Electron Microscopy

To quantify the frequency of granule–MT complexes in neutrophil cavitates or in mixtures of purified MTs and isolated granules, samples were examined in a JEOL 100C (JEOL USA, Peabody, MA) electron microscope and measurements of MT-attached granules per MT length were made. Samples

containing nontaxol-stabilized MTs were diluted into 1% glutaraldehyde solution to fix MTs before adsorption to carbon-coated parlodian grids. Samples containing taxol-stabilized MTs were applied directly to the grids. Grids were then stained with 2% uranyl acetate and examined in the electron microscope. All MTs and granules in selected grid squares were measured until a minimum of 400-μm polymer length (typically 25–35 MTs for each experimental point) were examined to quantify granule–MT associations in vitro and 100-μm polymer length (typically 35–40 MTs) to quantify granule–MT associations formed in vivo. Samples were also examined by scanning EM, after fixation with 1% glutaraldehyde, application to polylysine-coated glass coverslips, and postfixation with 2% osmium tetroxide. After gold coating, samples were viewed in a Hitachi, Ltd. E5741 scanning electron microscope.

Specific labeling of neutrophil granules was accomplished using antilactoferrin antibody to identify secondary granules and peroxidase staining to identify primary granules. Glutaraldehyde-fixed samples were prepared as described above. For lactoferrin labeling, granules were permeabilized with 0.01% Triton X-100 in 20 mM Tris, pH 7.4, for 30 s, washed with Pipes buffer, and incubated with anti-human lactoferrin antibody (Organon Teknika–Cappel Laboratories, Malvern, PA) at 100% humidity and 37°C for 1 h. This was followed by a 1-h incubation with protein A–gold and negative staining with 0.3% uranyl acetate. Primary granules were stained with *p*-phenylenediamine–distilled HCl and catechol following the Sigma Chemical Co. Procedure No. 390, and then counterstained with 0.1% uranyl acetate to permit identification of MTs.

MT-activated ATPase Activity Assay

MT-activated ATPase assays were performed on human neutrophil cytosol, isolated granules, and granule extracts using modifications of the method of Pollard and Korn (34). Neutrophils were disrupted by cavitation as described above and the granule-rich preparation was centrifuged at 81,000 *g*. The supernatant was removed to be assayed as "cytosol" and the pellet was resuspended in 100 mM Pipes, pH 6.9, containing 1.0 mM EGTA, 1.0 mM EDTA, 3.0 mM $MgCl_2$, 1 mM dithiothreitol, 1.0 mM PMSF, 0.2 mM leupeptin, and 0.2 μg/ml aprotinin (protein concentrations 0.3–1.2 mg/ml) to be assayed as "granules".

Neutrophil granules were separated from cytosolic proteins by column chromatography. Cavitates from human neutrophils (2–5 × 10^5 cells), prepared as above, were applied to a Sepharose 6B column (2 × 25 cm) and eluted with 100 mM Pipes buffer (PEM) containing 1 mM $MgSO_4$ and 1 mM EGTA. A mixed granule population eluted with the void volume ahead of the soluble cytosolic proteins. Granule-containing fractions were then pooled and an equal volume of PEM buffer with 2 M KCl and protease inhibitors was added. After extraction in this salt solution at 5°C for 30 min, the granules were centrifuged at 112,000 *g* for 30 min at 5°C. The supernatant was then desalted and concentrated by centrifugation in Centricon 30 microconcentrators (Amicon Corp., Danvers, MA).

Cytosol, granule, or extract samples (200 μl) were then mixed with 200 μl taxol-stabilized rat brain MTs (PC-purified tubulin, 0.5–2.0 mg/ml) or 200 μl granule buffer and warmed to 37°C. The ATPase reaction was started by addition of 40 μl of 50 mM γ-[^{32}P]-ATP (ICN Pharmaceuticals Inc., Irvine, CA) with a specific activity of 4,000 dpm/nmol. 200-μl samples were taken at 5, 15, or 30 min, and the reaction was terminated by extraction in a solution of silicotungstic acid (0.5 ml) and isobutanol/benzene (1:1 vol/vol) (2.0 ml) while adding 0.2 ml of ammonium molybdate (10%). 1.0 ml water was added to facilitate extraction and 0.5 ml of the organic phase was taken for analysis by scintillation counting. MT-activated ATPase activity was calculated by subtracting the intrinsic ATPase activity of the MTs (mean ± SD = 4.2 ± 2.7 nmol/min per mg, n = 7) added to granules or extracts from the total activity detected in reaction mixtures.

Procedures for SDS-PAGE, Immunoblotting, and Autoradiography

Conditions for SDS-PAGE were as previously described (29). Proteins were fractionated on 10% acrylamide-*N*,*N*'-diallytartardiamide gels, pH 8.8, or 10–15% gradient Phastsystem gels (LKB Instruments, Inc.–Pharmacia Fine Chemicals, Piscataway, NJ) and transferred to nitrocellulose paper (47). The papers were incubated in solutions containing antibody specific for *Drosophila* kinesin diluted 1/250 (gift of Dr. Jonathan Scholey, University of Colorado, Health Sciences Center, Denver, CO), washed in TBS with 0.05% Tween-20, and incubated with alkaline phosphatase–conjugated goat anti-rabbit immunoglobulin antibody, diluted 1/250. Antibody–antigen complexes were visualized after washing with TBS with 0.05% Tween-20 and 30 mM Tris, pH 8.0, by the addition of 1 mg/ml Naphthol AS-MX-phosphate and 2 mg/ml Fast Red–Texas Red salt. Purified bovine brain kinesin (gift of Dr. Douglas B. Murphy, The Johns Hopkins School of Medicine, Baltimore, MD) was used as a positive control for the procedure.

ATP-binding proteins were visualized by the method of Maruta and Korn (26). Neutrophil cytosol and extract fractions (30 μl) were incubated with [^{32}P]α-ATP (6 μCi/sample, 6,000 Ci/mmol) (New England Nuclear) at 4°C with 100 mM ATP added as a competitor to parallel samples. The samples were exposed to short wave UV (254 nm) for 40 min. Unbound radioactivity was removed by centrifuging the samples through G-25 columns (0.5 × 3.0 cm). The samples were then electrophoresed as above, and the gels dried and exposed to XOMAT-AR film (Eastman Kodak Co., Rochester, NY).

Results

Granule Movements in Living Neutrophils

In initial studies we confirmed the findings of others (3) that isolated human neutrophils possess radial MT arrays that may be visualized by immunofluorescence (data not shown). These MTs form a network which is less extensive than that seen in other cell types such as kidney and mouse 3T3 cells (32). Nonetheless, MTs appear to extend to the periphery of the cell.

In video-enhanced microscopy studies of living, spread neutrophils, we also confirmed the early reports of Bessis and co-workers (4, 35) that linear, to-and-fro radial movements of granules occur along paths that correspond to the radial distribution of MTs in these cells. These linear movements were easily distinguishable from the prominent, short range ($\leq$0.1 μm), agitated movements that characterize most granules at any given moment. Illustrated in Fig. 1 are representative granule movements of this type which had a rate of 1–3 μm/s for distances equivalent to 1/4–1/3 of the cell width. Such movements occurred frequently, with granules moving both from the periphery to the interior of the cell (as illustrated in Fig. 1) and also from the interior to the periphery of the cell. Cells were often observed to display the movement of multiple granules in tandem on the same track as well as single granules moving on different tracks. Comparison of fMLP-stimulated cells with unstimulated cells revealed no qualitative differences, although it is probable that none of the neutrophils, once they had adhered to the glass slide, were in a nonactivated, resting state.

Isolation of Granule–MT Complexes from Human Neutrophils

It was anticipated that cytoplasmic granules and MTs might form close associations in neutrophils if MTs direct the translocation of granules in these cells. Indeed, the presence of native tubulin in granule-enriched fractions isolated from human neutrophils has been recently reported (30). We found that granule–MT complexes could be recovered from intact neutrophils when the cells were treated with 1–10 μM taxol to stabilize MTs, and were then disrupted by either nitrogen bomb cavitation or sonication, and examined by transmission EM. As has been reported by Roberts et al. (37), incubation of intact neutrophils with high concentrations of taxol ($\geq$10 μM) was found to result in MT bundling; however, lower concentrations (1–2 μM) of taxol were sufficient to stabilize MT structure, without polymer bundling. Fig. 2 shows examples of granule–MT complexes re-

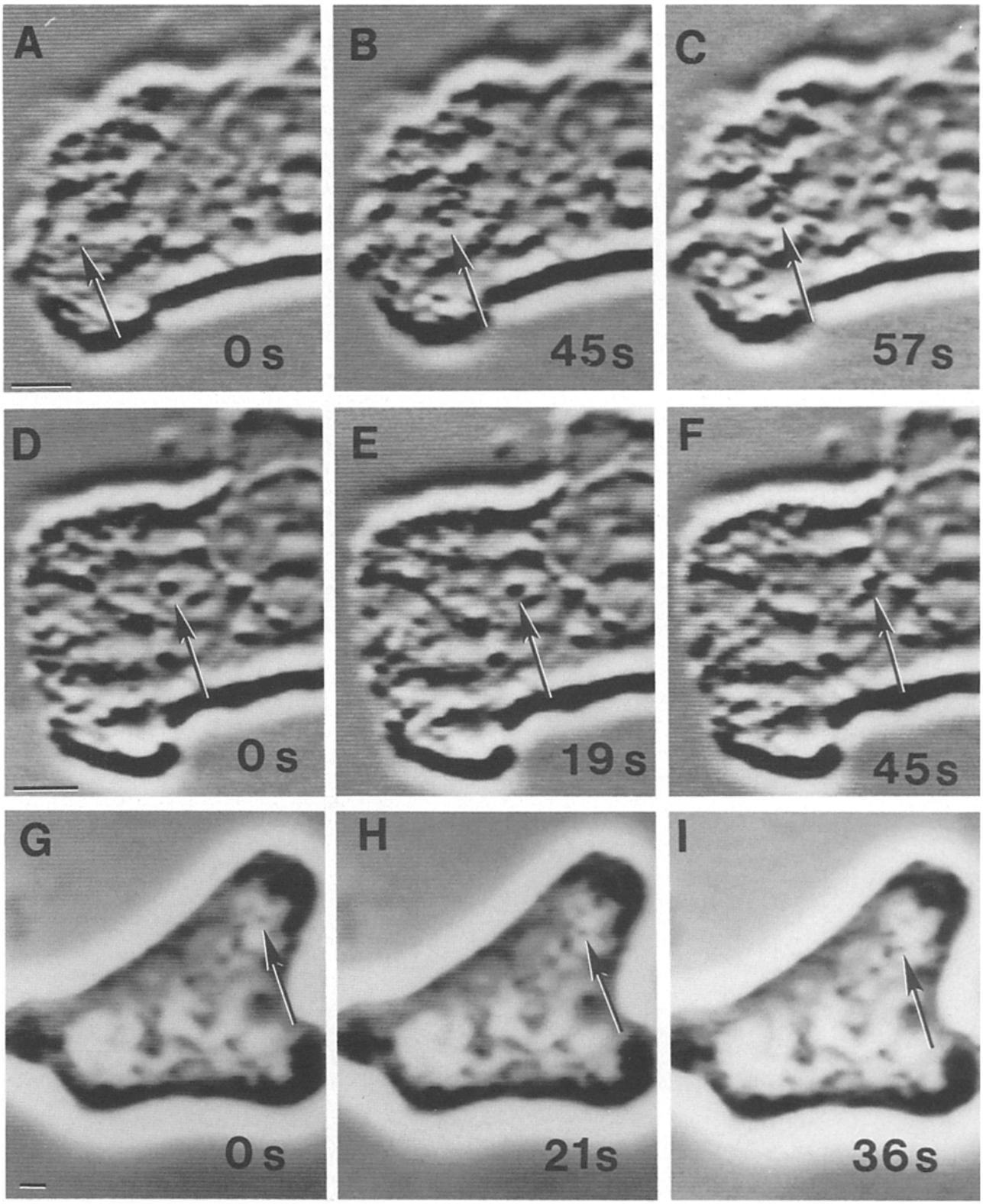

Figure 1. Linear, nonrandom movements of cytoplasmic granules in living neutrophils. The movement of an individual granule was traced in a spread neutrophil in each of the three sequences by DIC microscopy (*A–F*) and phase-contrast microscopy (*G–I*). Arrows mark the successive positions of these individual moving granules. Bars, 2.5 μm.

covered from resting neutrophils and from cells incubated briefly with fMLP (10^{-7} M).

Identification of organelles (300–600 nm in width) as primary and secondary granules was confirmed by histochemical and immunological labeling techniques illustrated in Fig. 3. Both peroxidase-positive primary granules as well as secondary granules, which labeled with anti-human lactoferrin antibody coupled to protein A–gold particles, were found at-

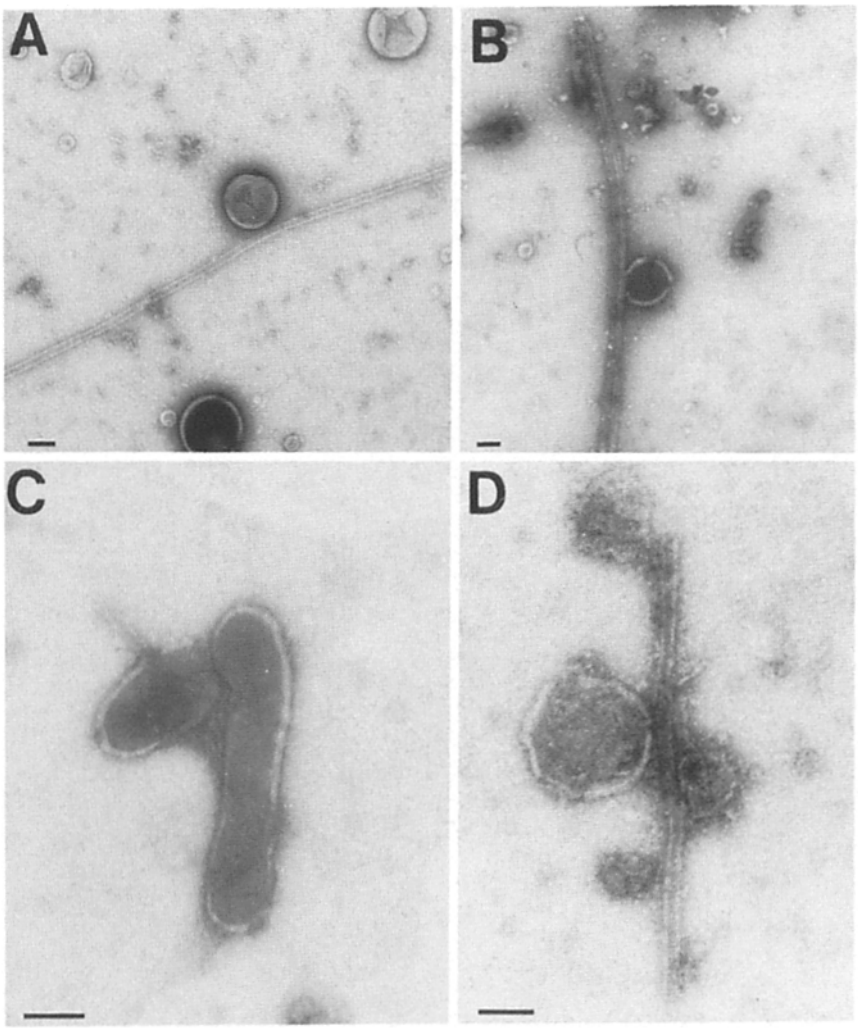

Figure 2. Granule–MT complexes isolated from resting and fMLP-stimulated neutrophils. Neutrophils were incubated in the absence (*A* and *B*) or presence (*C* and *D*) of fMLP (10^{-7} M) for 5 min at 37°C; cells were then incubated with 1 μM taxol for 5 min at 37°C and disrupted by sonication. Sonicates were adsorbed to carbon-coated grids, stained with uranyl acetate, and examined by EM. Bars, 100 nm.

tached to MTs in preparations of disrupted, taxol-treated neutrophils. With the negative-staining technique used to examine granule–MT complexes by transmission EM, the lactoferrin-positive secondary granules displayed an electron-dense center with a clear boundary. These granules were also pleomorphic, including both round and elongated forms, and did not label with peroxidase (Fig. 3, *E* and *G*). This staining characteristic of secondary granules was confirmed when purified granules preparations were examined (see Fig. 7 below). Granules that did not label with antilactoferrin antibody (Fig. 3 *C*) were more uniformly round with electron-lucent centers, and this negative-staining characteristic matched that of purified primary granules (Fig. 7). In addition to granules, smaller vesicles (100–300 nm), which were lactoferrin and peroxidase negative, were also recovered in preparations of disrupted neutrophils and were occasionally associated with MTs. The identity of these smaller vesicles, which could represent endosomes or plasma membrane vesicles generated during cell disruption procedures, was not determined in our studies, and these vesicles were not included in the quantitative measurements of granule–MT interactions described below.

It was evident that granule–MT complex formation in neutrophils is a regulated event. Both the relative recoveries of these complexes (e.g., the percent of granules attached to MTs vs. unattached) and the numbers of granules attached to individual MTs in complexes (e.g., the number of granules/MT length) were increased two- to threefold in fMLP-stimulated neutrophils (Fig. 4, *A* and *B*). Increased granule–MT complex formation was stimulated by fMLP at concentrations as low as 10^{-8} M and was maximal at 10^{-7}–10^{-6} M. Furthermore, as shown in Fig. 4 *C*, fMLP-stimulated, granule–MT complex formation occurred very rapidly. Increased complex formation was detectable by 30 s of incubation of neutrophils with fMLP (10^{-7} M) and reached a plateau at ~2 min. In addition, fMLP-stimulated formation of granule–MT complexes was inhibited in a dose-responsive fashion by concurrent incubation of the cells with dibutyryl cAMP (10^{-5} to 10^{-8} M) (Fig. 5).

The appearance of MTs recovered from neutrophils activated with fMLP was qualitatively different from that of MTs recovered from resting cells. MTs from activated neutrophils were partially coated with small (~10-nm) globular particles, which were only infrequently observed on MTs recovered from resting cells (Fig. 6). These particles sometimes appeared to be distributed in a spiral pattern on MTs (Fig. 6 *B*) similar to those described for the decoration of brain MTs by MAP2 (13, 20).

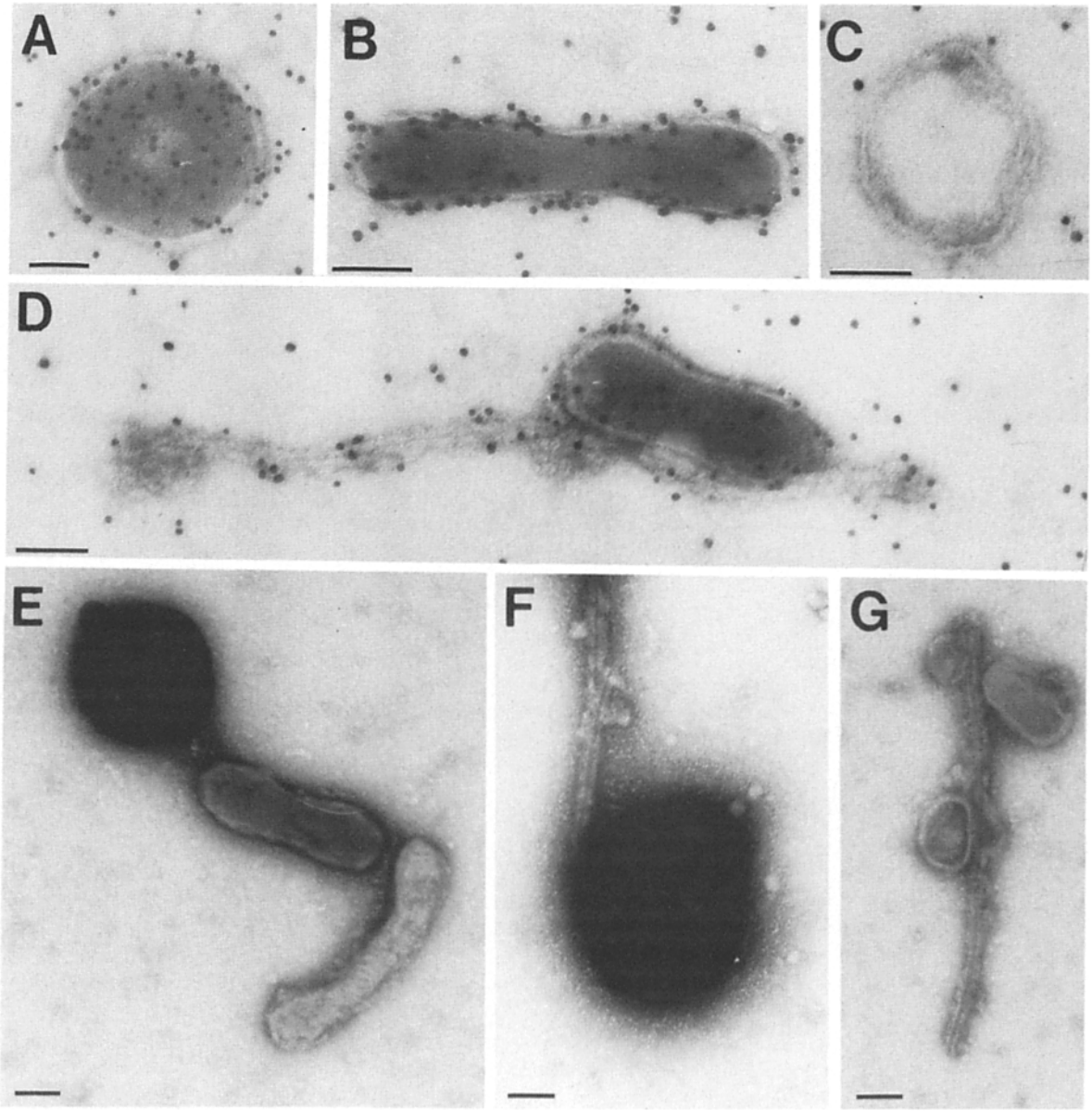

Figure 3. Identification of peroxidase-positive, primary and lactoferrin-positive, secondary neutrophil granules. (*A* and *B*) Binding of anti-human lactoferrin antibody-linked gold particles to detergent-treated secondary granules counterstained with uranyl acetate. (*C*) Lactoferrin-negative granule. (*D*) Lactoferrin-positive, secondary granule attached to MT. (*E*) Peroxidase-positive and -negative neutrophil granules counterstained with uranyl acetate. (*F* and *G*) Attachment of peroxidase-positive and -negative granules to MTs. Bars, 100 nm.

Formation of Granule–MT Complexes in a Cell-free System

To define the components involved in mediating granule–MT interactions, we attempted to reproduce the complexes recovered from disrupted cells by using a cell-free, reconstituted system. Because of the low tubulin content of human neutrophils, chicken or rat brain tubulin was used to generate purified MTs. Granules were isolated from human neutrophils as either a mixed granule preparation with cytosol or were further separated by discontinuous sucrose gradients into high and low density (primary and secondary) granule-enriched fractions (Fig. 7). When either mixed or gradient-isolated granules were incubated with MTs and then examined by EM (Fig. 8) or by video-enhanced microscopy, granule–MT complexes were observed. There was no clearly demonstrable difference in the binding to MTs by high density primary granules and low density secondary granules; both granule types appeared to interact with MTs with a similar avidity. In 10 experiments, mean granules per MT length (G/MT) values were found to be 0.05 ± 0.05 (mean ± SD) for primary granules and 0.11 ± 0.06 (mean ± SD) for secondary granules (values which were not statistically different). We also found that these interactions were not specific for a particular source of MTs, since similar levels of interactions were also observed with reconstituted chicken erythrocyte MTs and with the organized MT structures of isolated *Chlamydomonas* axonemes. Moreover, the presence

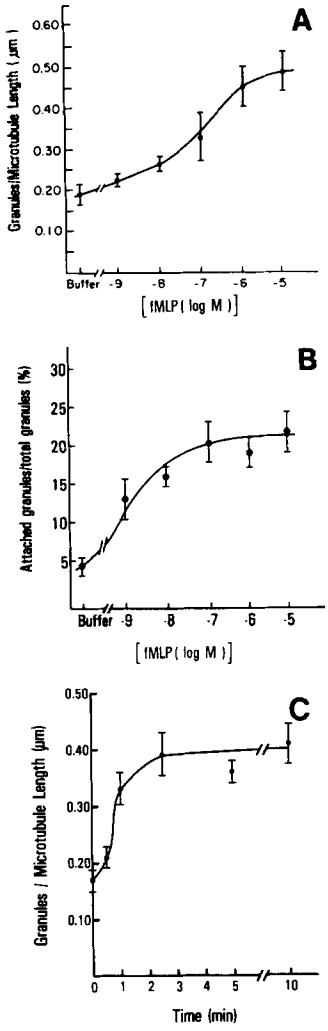

Figure 4. Stimulation of neutrophil granule–MT complex formation in cells incubated with varying concentrations of fMLP (10^{-9}–10^{-5} M). (*A*) Granule–MT interactions expressed in terms of attached granules per MT polymer length (μm). (*B*) Granule–MT interactions expressed as percent of total granules attached to MTs. (*C*) Kinetics of fMLP-stimulated granule–MT interactions. Results represent mean values ± SEM for four replicate studies.

or absence of cytosol in organelle preparations did not affect the formation of these complexes.

Examination of reconstituted granule–MT complexes at high magnification revealed the presence of small globular particles similar to those observed on MTs recovered from intact neutrophils (Fig. 8 *C*). In some cases, these structures appeared to form bridges between granules and MTs. Decoration of MTs with particles was markedly enhanced by the addition of sodium orthovanadate (10 μM) to granule–MT mixtures (Fig. 8 *D*).

Modulation of Granule–MT Interactions by Inhibitors of ATPases

To determine if the formation of granule–MT complexes, recovered from disrupted neutrophils and reproduced in the cell-free system, might be mediated by an ATP-dependent, force-generating protein or proteins analogous to kinesin (48) or dynein (31, 33), we examined the formation of such complexes in the presence of various agents known to affect ATPase activity. We first used the general enzymatic inhibitor, *N*-ethylmaleimide. This sulfhydryl alkylating agent at concentrations of 1–5 mM completely eliminated G/MT interactions in the reconstituted system (Fig. 9 *A*). In contrast, the addition of sodium orthovanadate at micromolar concentrations increased these interactions as much as fourfold (Fig. 9 *B*) with maximum effects observed at ≥1.0 μM vanadate. Moreover, as noted above, these concentrations of vanadate-induced MT decoration similar to that observed in preparations of disrupted neutrophils stimulated by fMLP. AMP-PNP, a nonhydrolyzable analogue of ATP, on the other hand, decreased G/MT interactions when added to incubation conditions at concentrations of 0.1–5.0 μM (Fig. 9 *C*).

The effects of vanadate and AMP-PNP on granule–MT complex formation involving sucrose gradient–isolated granules and in vitro–polymerized MTs are shown in Table I. Unlike the results of experiments involving granule–MT complexes isolated from disrupted cells or those experiments which used mixed granule preparations in which the granule concentration could be tightly controlled, the values for the baseline G/MT in experiments using gradient-isolated granules varied from experiment to experiment due to the variation in the concentration of the purified granules. For this reason, the effects of the ATPase inhibitors were only compared to the corresponding control values for each experiment. AMP-PNP reproducibly reduced G/MT values and demonstrated slightly greater inhibitory effects on the interactions of low density, secondary granules with MTs than on those observed with high density, primary granules. The addition of 1 μM vanadate to the cell-free system caused an increase in G/MT values, and this effect was also more prominent with secondary granules.

We also examined the relative numbers of granules that were attached vs. those unattached to MTs under these experimental conditions. For these assessments all granules and all MTs (up to a cumulative polymer length of 400 μm; typically 25–35 MTs) were counted in selected electron microscope grid squares. In four replicate studies, a mean of 38 ± 9% (±SD) of granules were attached to MTs under control conditions, while AMP-PNP at concentrations of 0.5, 1.0, and 5.0 mM reduced the percentage of attached granules to 28, 24, and 22%, respectively.

Since AMP-PNP caused a decrease in granule–MT interactions, we suspected that high concentrations of ATP might also decrease these interactions. Consistent with this possibility, granule–MT complexes formed in the presence of low

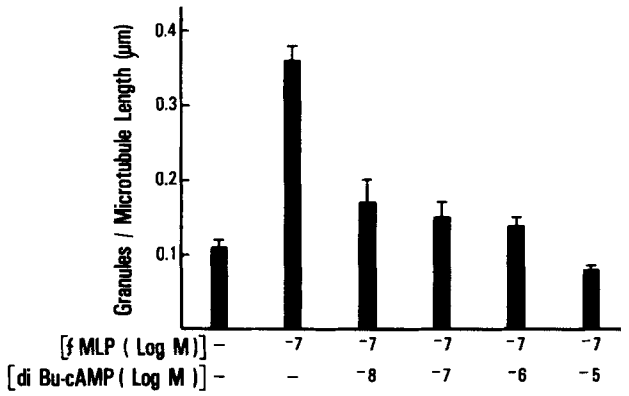

Figure 5. Inhibition of fMLP-stimulated granule–MT complex formation by dibutyryl cAMP. Results represent mean values ± SEM for three replicate studies.

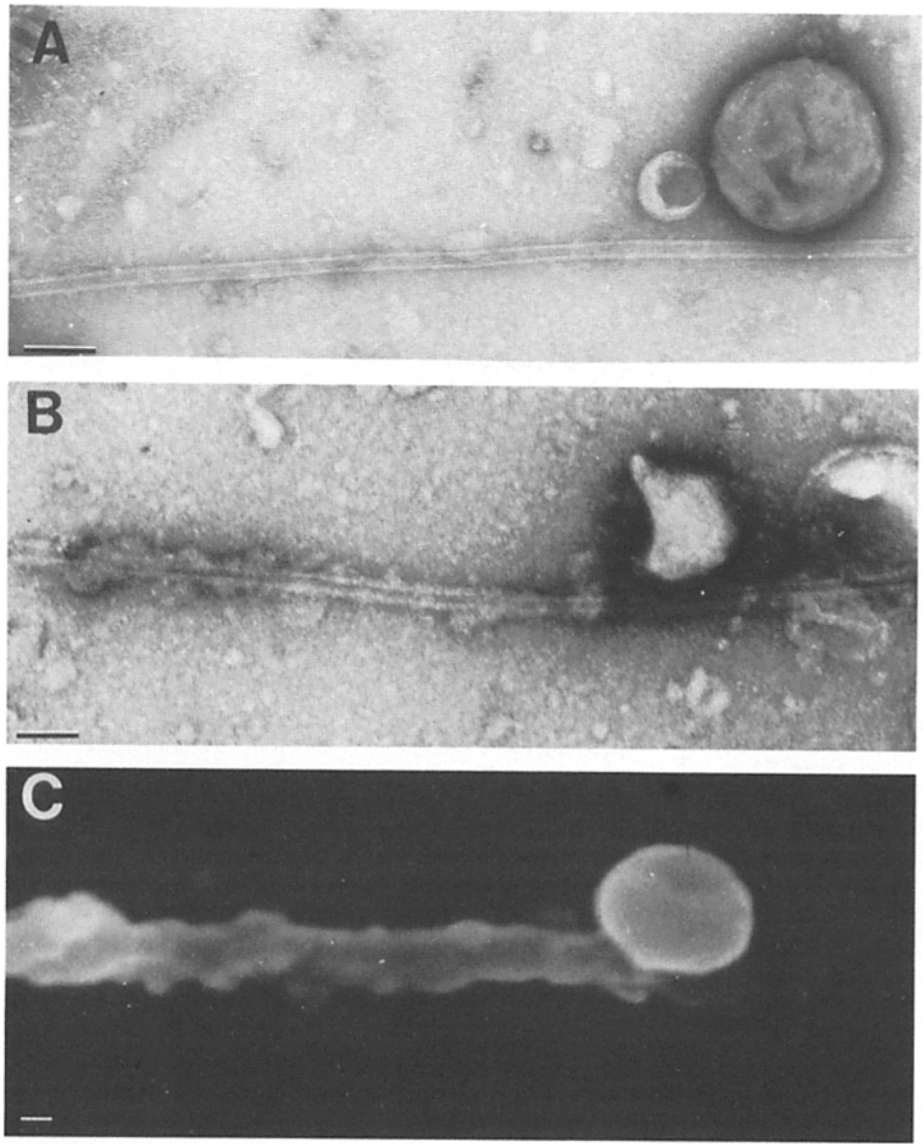

Figure 6. Decoration of MTs recovered from fMLP-stimulated neutrophils. (*A*) Uranyl acetate–stained MT from resting neutrophil. (*B*) MT from neutrophil incubated with 10^{-7} M fMLP. The lefthand end of the MT illustrates an example of MT decoration in a spiral pattern. (*C*) Gold-coated MT–granule complex visualized by scanning EM. Bars, 50 nm.

(10–30 μM) concentrations of ATP displayed higher and more variable G/MT values than did those formed in the presence of high (1.0 mM) concentrations of ATP (G/MT = 0.15 $\pm$ 0.09 for low ATP vs. 0.03 $\pm$ 0.02 for high ATP).

Localization of an MT-activated ATPase to Neutrophil Granules

When neutrophils were disrupted by cavitation, separated into cytosol and granule fractions, and then assayed for the presence of ATPase activity, we found that the ATPase activities detectable in both cell fractions were increased when these fractions were allowed to interact with MTs. However, the greatest MT-mediated increase in ATPase activity was observed with granule fractions. The specific activity of cytosolic ATPase, which was 9.9 $\pm$ 0.9 nmol/min per mg (mean $\pm$ SEM, n = 4) initially, increased by 10% to 10.9 $\pm$ 1.1 nmol/min per mg protein when assayed in the presence of taxol-stabilized MTs (total ATPase activity minus ATPase activity of MTs alone). In contrast, the ATPase activity associated with intact, isolated granules was increased an average of 15-fold (from 2.9 $\pm$ 0.7 to 41.0 $\pm$ 12.7 nmol/min per mg protein, n = 4; Table II) when MTs were present in reaction mixtures. Treatment of column-separated neutrophil granules with PEM buffer containing 2 M KCl yielded extracts enriched in the MT-activated ATPase. With PC-purified rat brain MT, the specific activity of ATPase in these extracts was increased >100-fold (Table II), reflecting a ≥10-fold purification of the MT-activated ATPase associated with the neutrophil granules.

When we examined these extracts with an antibody against *Drosophila* kinesin, we found that they contained a polypeptide of ~115–120 kD which immunologically cross-reacted with this antibody in Western blots (Fig. 10, *A* and *B*). To characterize this protein further, we also assayed these extracts for proteins with ATP-binding activity. Autoradiogra-

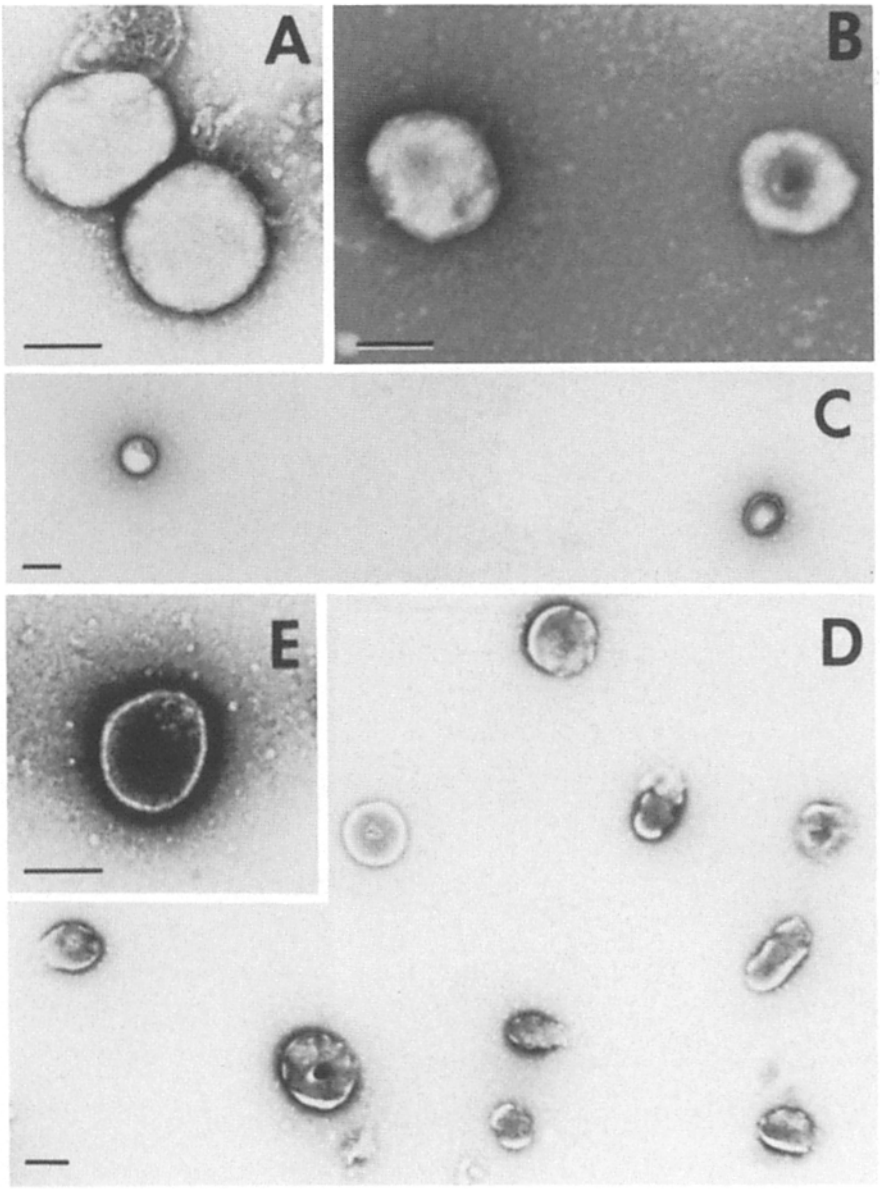

Figure 7. Neutrophil granules isolated by discontinuous sucrose gradients (see Materials and Methods). (*A–C*) Primary, myeloperoxidase-containing granules sedimenting at the 45/60% sucrose interface. (*D* and *E*) Secondary, peroxidase-negative granules sedimenting at the 30/45% sucrose interface. Samples were prepared for EM as in Figs. 2, 3, and 6. Bars, 200 nm.

phy of samples which had been incubated with radiolabeled ATP, and then exposed to UV irradiation to covalently link ATP to its binding sites (26), demonstrated a band at ~115–120 kD and competition with nonlabeled ATP eliminated this band (Fig. 10 *C*).

Discussion

In these studies we have investigated interactions that occur between MTs and cytoplasmic granules in both resting and activated human neutrophils. We confirmed previous reports that MTs are present in neutrophils in a radial configuration that is appropriate both for the translocation of granules from the cell center to the periphery and for the translocation of endosomes and phagosomes centrally from the cell surface (3, 4, 42). We also confirmed earlier reports that granules within spread, living neutrophils move along linear, radial tracts that have an orientation similar to the MT array in these cells. When neutrophils were treated with taxol to stabilize MTs and then disrupted, many of the cytoplasmic granules recovered from the cells were found to be attached to MTs. Furthermore, the formation of granule–MT complexes in neutrophils was found to be a regulated event that increased with activation of the cells. When neutrophils were stimulated with fMLP, the number of granule–MT complexes relative to the total number of granules recovered was increased as were the numbers of granules attached to individual MT fragments. Increased granule–MT complex formation occurred rapidly after exposure of neutrophils to the chemotactic peptide; it was detectable within 30 s and maximal by 2 min. This stimulus-responsive formation of granule–MT complexes was also dependent upon the con-

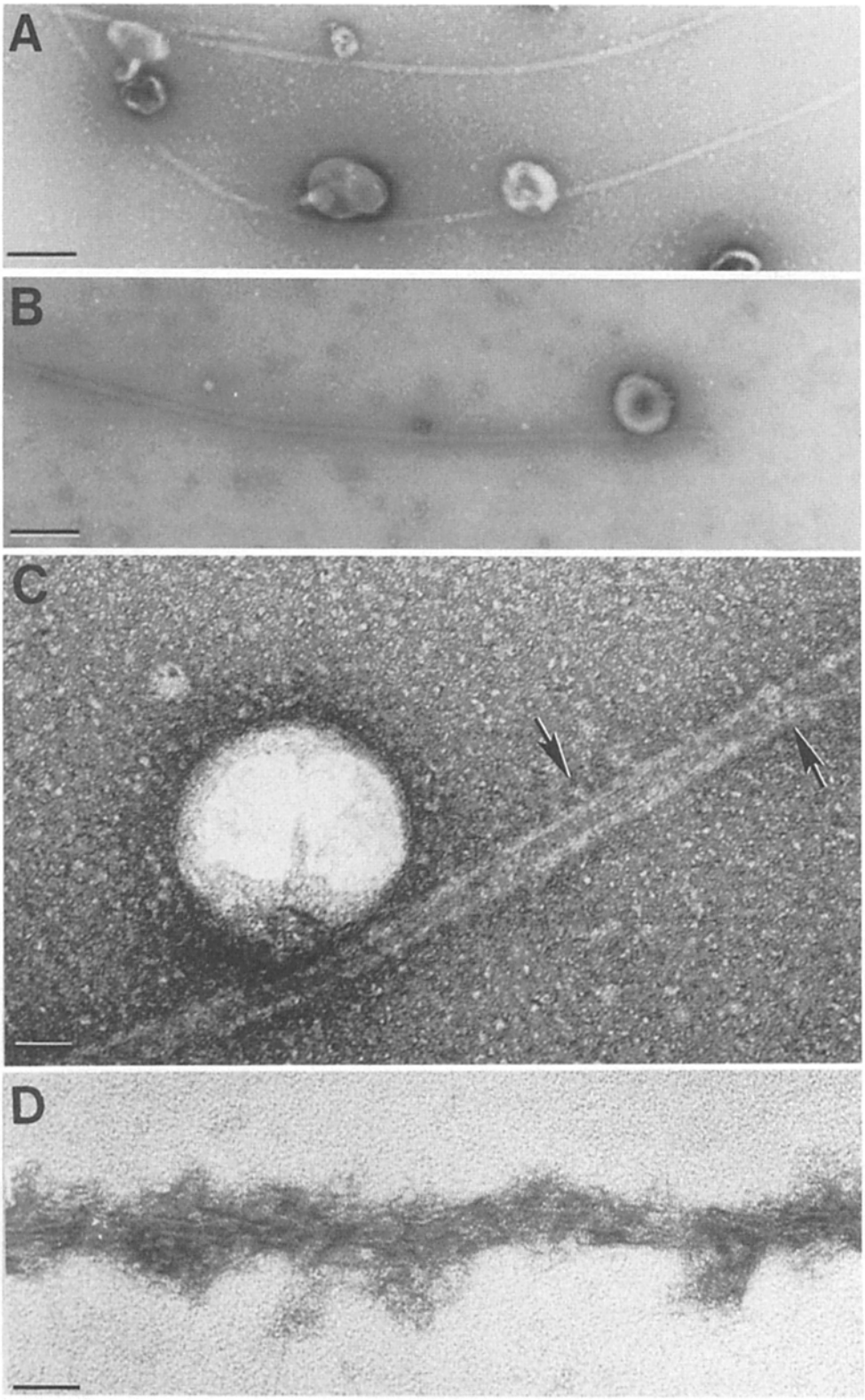

Figure 8. Interactions of isolated neutrophil granules with purified chicken brain MTs. (*A* and *B*) Uranyl acetate–stained, reconstituted granule–MT complexes. (*C*) High magnification view of reconstituted granule–MT complex; arrows indicate ~10-nm MT-associated particles. (*D*) Heavy microtubule decoration after incubation with neutrophil cytosol and vanadate (10 μM). Bars: (*A* and *B*) 200 nm; (*C* and *D*) 50 nm.

centration of fMLP and was maximal at concentrations of $\geq 10^{-7}$ M which are similar to those required for stimulation of granule exocytosis by this peptide (8, 41). On the other hand, stimulus-dependent, granule–MT complex formation was inhibited by incubation of neutrophils with dibutyryl cAMP, an agent which has been shown to inhibit neutrophil degranulation (52, 57, 58), at concentrations of 10^{-7}–10^{-5} M.

The granule–MT complexes recovered from neutrophils were morphologically similar both to complexes of MTs and storage granules isolated from pancreatic islet cells (10) and

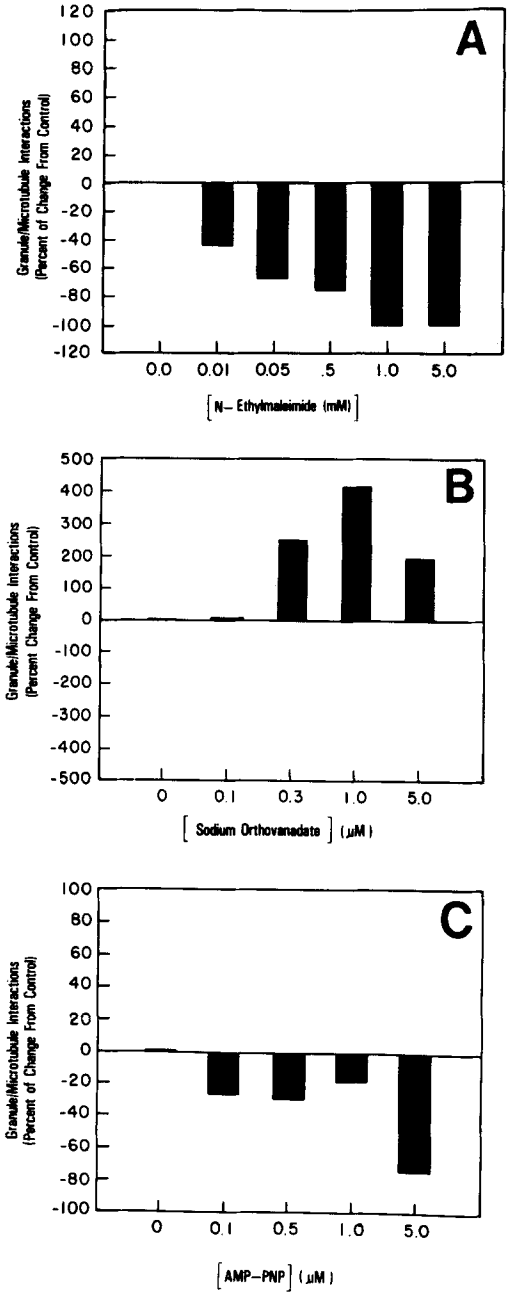

Figure 9. Effects of ATPase inhibitors on granule–MT interactions. Mixed granules in 100 mM Pipes buffer, pH 6.9, containing 10 μM ATP were mixed with taxol-stabilized chicken brain MTs in the presence of various concentrations of (*A*) *N*-ethylmaleimide, (*B*) sodium orthovanadate, or (*C*) AMP-PNP, and incubated for 10 min at 37°C before preparation for EM. Results from representative studies are shown.

to MT–vesicle complexes which have been observed in neuronal cells (36). However, in neither of these other cell systems has the formation of granule or vesicle–MT associations been shown to be influenced by extracellular stimuli.

We have also demonstrated that neutrophil granule–MT interactions can be reproduced in a reconstituted, cell-free system. We anticipated that this might be possible since attachment of vesicles recovered from other types of cells (neuronal cells, in particular [2, 7, 22, 33, 43, 49]) to MTs has been successfully reproduced in vitro. Furthermore, we anticipated that neutrophil granule–MT associations might involve specific ATPases, such as kinesin (48) and/or cytoplasmic dynein (33), which in other cell types have been shown to mediate vesicle movement along MTs (9, 33, 40, 50, 51).

In our studies, isolated neutrophil granules were found to form complexes with purified MTs polymerized in vitro from tubulin derived from a variety of different sources (e.g., avian, bovine, and rodent brain tissue, as well as avian erythrocytes). Neutrophil granules were also found to form complexes with the organized MTs of isolated *Chlamydomonas* axonemes. Gradient-purified primary and secondary granules had a similar propensity to form MT attachments, and agents which are known to block or modify ATPase-dependent processes (sodium orthovanadate, AMP-PNP, *N*-ethylmaleimide) were found to modulate these attachments. Moreover, neutrophil granules were found to be enriched with an MT-activated ATPase activity which could be isolated from intact granules and partially purified by salt extraction, and it was found that these extracts contained a 115–120-kD protein or proteins which cross-react with an antikinesin antibody and bind ATP. However, the characteristics of pharmacologic inhibition and enhancement of granule–MT interactions in vitro suggests that an ATPase or ATPases other than kinesin might be involved in these interactions. Cytoplasmic dynein, which has been shown to mediate retrograde movement of vesicles along MTs, is sensitive to vanadate and *N*-ethylmaleimide while kinesin is largely unaffected by these agents (50).

Given the observations of others concerning vesicle–MT complex formation in melanophores (27, 28, 38), pancreatic cells (10), pituitary cells (46), and neuronal cells (36), it would appear likely that the regulated interactions of neutrophil granules with MTs, documented in our studies, represent a component of an active MT-based vesicle translocation system in these cells. Indeed, previous studies of MT morphology and function in human neutrophils support the conclusion that granule mobilization in neutrophils is an MT-based activity. Electron micrographs of thin-sectioned and whole-mount cell preparations have revealed close associations between granules and MTs (8, 16). Moreover, in cells stimulated with fMLP or zymosan-treated serum, invaginations of the plasma membrane have been observed to arise during the sequential fusion of cytoplasmic granules with the cell surface and to be oriented along MTs radiating from the centrosome (8, 16). Furthermore, ultrastructural examinations of stimulated neutrophils have shown that increases in the secretion of granule contents correlate with increases in the numbers of MTs observed in these cells (16, 17), and disruption of the MT network in neutrophils by colchicine or vinblastine has been shown to decrease granule exocytosis (55, 58). Additional evidence for an MT-based vesicle translocation system in phagocytic cells has been provided by the recent studies of Swanson et al. (44), who have observed that the tubular lysosomal system of mouse peritoneal macrophages becomes associated with the MT network after treatment of the cells with phorbol esters.

As noted above, microscopic examination of living neutrophils reveals extended, linear movements of individual granules between the center and periphery of cells. Several lines

Table I. Effects of ATPase Inhibitors on Granule–MT Interactions

A. Treatment	Experiment	Granule type*	Control (granules/μm MT)	Vanadate‡ (granules/μm MT)	Change
					%
Sodium orthovanadate (1.0 μM)	1	Primary	0.02	0.043	+253
		Secondary	0.02	0.067	+394
	2	Primary	0.075	0.096	+128
		Secondary	0.094	0.19	+202
	3	Primary	0.12	0.04	−66
		Secondary	0.05	0.08	+160

B. Treatment	Experiment	Granule type*	Control (granules/μm MT)	AMP-PNP‡ (granules/μm MT)	Change
					%
AMP-PNP (0.5 mM)	1	Primary	0.075	0.044	−41
		Secondary	0.094	0.018	−81
	2	Primary	0.12	0.082	−32
		Secondary	0.05	0.02	−64

* Human neutrophils were isolated as described in Materials and Methods and disrupted by nitrogen cavitation. Cavitates were centrifuged at 230 *g* for 10 min to remove unbroken cells and nuclei, and then layered onto discontinuous sucrose gradients for separation into a low density, secondary granule-enriched fraction and a high density, primary granule-enriched fraction.

‡ Granules were diluted to 0.1–0.2 mg/ml protein content with 100 mM Pipes buffer and mixed with taxol-stabilized MTs. Vanadate or AMP-PNP was added from concentrated stocks to give the desired concentration. After a 5-min incubation at 37°C, samples were prepared for electron microscopy.

of evidence suggest that these linear movements represent an active vesicle translocation system directed by MTs. Studies by Luby-Phelps et al. (23), using fluorescence recovery after photobleaching techniques with sized fluorescein-labeled Ficoll particles in Swiss 3T3 cells, have indicated that, due to the high viscosity of the cytoplasm, large cellular organelles should be relatively immobile without active interactions with the cytoskeleton. Also, the rates of linear granule movements in spread neutrophils measured in our studies are similar to those of the MT-directed movements of organelles in other cell systems (33, 50).

Neutrophils are likely candidates to possess a regulated, MT-based vesicle translocation system, for it has long been recognized that granule mobilization is a prominent compo-

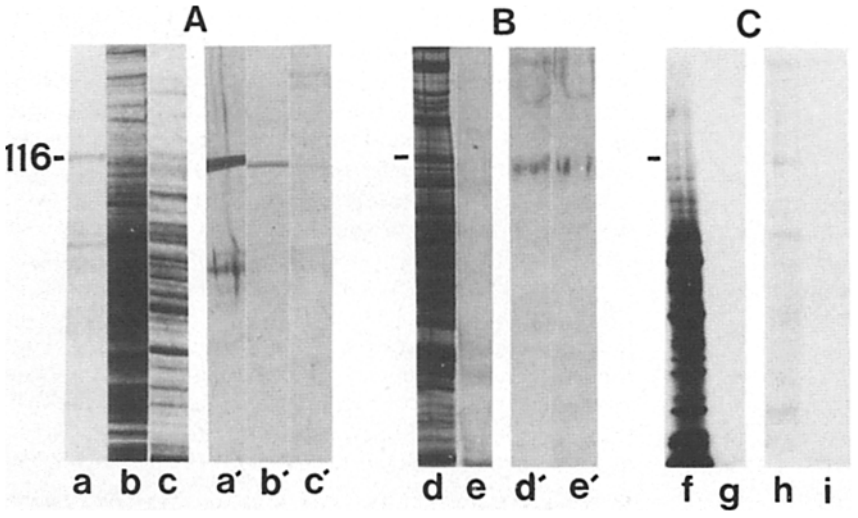

Figure 10. Identification of a kinesin-like, ATP-binding protein in neutrophil extracts. (*A* and *B*) Immunoblot analysis of neutrophil extracts with polyclonal rabbit anti-*Drosophila* kinesin antibody. Purified bovine brain kinesin (lanes *a* and *a'*), bovine brain extract (lanes *b* and *b'*), and neutrophil whole cell extract (lanes *c* and *c'*) were electrophoresed (silver stained in lanes *a*, *b*, and *c*) and then immunoblotted on nitrocellulose paper (lanes *a'*, *b'*, and *c'*). Immunologically cross-reactive bands were visualized with an alkaline phosphatase–conjugated goat anti-rabbit antibody as described in Materials and Methods. Bovine brain extract (lanes *d* and *d'*) and neutrophil granule extract (lanes *e* and *e'*) were fractionated by electrophoresis on a 10–15% Phastsystem gradient gel and blotted as above. (*C*) Autoradiograms of ATP-binding proteins photoaffinity labeled with α-[^{32}P]ATP. Neutrophil cytosol prepared in the absence (lane *f*) and presence (lane *g*) of 100 mM ATP and neutrophil granule extracts prepared in the absence (lane *h*) and presence (lane *i*) of 100 mM ATP were incubated with [^{32}P]ATP and irradiated with 254-nm UV light. Samples were then applied to a 10% SDS-acrylamide gel, fractionated by electrophoresis, and autoradiographed. The electrophoretic migration position of β-galactosidase (116 kD) is indicated.

Table II. MT-enhanced ATPase Activity in Human Neutrophil Granule Preparations

	Without MTs	With MTs*	Fold increase
	nmol/min/mg	*nmol/min/mg*	
Granule preparation 1‡	3.0	11.1	3.7
Granule preparation 2	1.0	21.0	21.0
Granule preparation 3	4.6	70.8	15.4
Granule preparation 4	3.1	61.2	19.7
Granule extract§	6.5	686.4	105.6

* Taxol-stabilized rat brain MTs were polymerized for 45 min at 37°C before addition to the samples. The assay was initiated by the addition of [^{32}P]ATP. ATPase values of granule preparations in the presence of MTs represent total ATPase activities minus ATPase activity of MTs alone (mean ± SD = 4.2 ± 2.7 nmol/min/mg).

‡ Human neutrophils were disrupted by nitrogen cavitation and granule-rich preparations were isolated as described in Materials and Methods, pelleted, and resuspended in 100 mM Pipes, pH 6.9, containing 1.0 mM EGTA, 1.0 mM EDTA, 3.0 mM $MgCl_2$, 1.0 mM PMSF, 0.2 mM leupeptin, and 0.2 μg/ml aprotinin for assays of ATPase activity.

§ Neutrophil cavitates were applied to a Sepharose 6B column to separate granules from soluble proteins. The granules were pooled and incubated with an equal volume of PEM buffer containing 2 M KCl. The samples were then centrifuged and the supernatants were desalted, concentrated, and assayed for ATPase activity.

nent of the functional response of these cells to phlogistic and phagocytic stimuli. While our studies clearly demonstrate that granule–MT interactions are stimulated in neutrophils as a consequence of cell activation, the molecular basis for these regulated interactions remains to be defined.

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