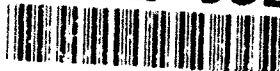


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13. ABSTRACT (Maximum 200 words) Neutrophil activation by specific stimuli is associated with an increased enzymatic addition of tyrosine to tubulin α -subunits. This increased tyrosine uptake into tubulin in activated neutrophils reflects an increase in the proportion of cellular tubulin which is tyrosinated rather than simply an increase in the turnover of tyrosinated subunits. The increased accumulation of tyrosinated tubulin was also found to follow an initial depletion of tyrosinated tubulin and concomitant increase in de-tyrosinated tubulin following stimulation of neutrophils with fMLF. These rapid changes in the relative content of tubulin isoforms in the cells were not associated with the formation or disappearance of microtubule microdomains composed of only one form of tubulin. Under conditions of fMLF-stimulated exocytosis there is an increased binding of neutrophil granules to endogenous microtubules. Since neutrophil activation by fMLF is associated with increased tyrosination of α -tubulin subunits, rapid changes in the levels of tyrosinated tubulin in the microtubules of activated neutrophils might have a role in the regulation of granule-microtubule interactions. When the binding of purified neutrophil granules to reconstituted rat brain microtubules containing approximately 50% tyrosinated tubulin was compared with granule binding to microtubules that contained no detectable tyrosinated tubulin, granule-microtubule associations were favored by de-tyrosinated vs. tyrosinated tubulin. These findings indicate that interactions between cytoplasmic granules and microtubules in activated neutrophils may be modulated by rapid changes in the relative content of de-tyrosinated and tyrosinated tubulin in the microtubule network of the cell.					
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Rapid and Reversible Tubulin Tyrosination in Human Neutrophils Stimulated by the Chemotactic Peptide, fMet-Leu-Phe

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Neutrophil activation by specific stimuli, such as the oligopeptide chemotactic factor fMet-Leu-Phe (fMLF), is associated with an increased enzymatic addition of tyrosine to tubulin α -subunits, as measured by ^{14}C tyrosine uptake. In studies using immunoblots we have found that this increased tyrosine uptake into tubulin in activated neutrophils reflects an increase in the proportion of cellular tubulin that is tyrosinated rather than simply an increase in the turnover of tyrosinated subunits. However, the increased accumulation of tyrosinated tubulin was also found to follow an initial depletion of tyrosinated tubulin and concomitant increase in detyrosinated tubulin between 0 and 60 sec following stimulation of neutrophils with fMLF. Immunogold electron microscopy studies of intact microtubules recovered from activated neutrophils demonstrated that these rapid changes in the relative content of tubulin isoforms in the cells were not associated with the formation or disappearance of microtubule microdomains composed of only one form of tubulin. Previously, we have shown that under conditions of fMLF-stimulated exocytosis there is an increased binding of neutrophil granules to endogenous microtubules. Since neutrophil activation by fMLF is associated with increased tyrosination of α -tubulin subunits, we speculated that rapid changes in the levels of tyrosinated tubulin in the microtubules of activated neutrophils might have a role in the regulation of granule-microtubule interactions. When the binding of purified neutrophil granules to reconstituted rat brain microtubules containing approximately 50% tyrosinated tubulin was measured by electron microscopy and compared with granule binding to microtubules that contained no detectable tyrosinated tubulin, granule-microtubule associations were found to be significantly favored by detyrosinated vs. tyrosinated tubulin. These findings indicate that interactions between cytoplasmic granules and microtubules in activated neutrophils may be modulated by rapid changes in the relative content of detyrosinated and tyrosinated tubulin in the microtubule network of the cells. © 1993 Wiley-Liss, Inc.

Granule mobilization and degranulation are important aspects of neutrophil function in acute inflammation and host-defense against microbial infection (Wright, 1982). There is compelling evidence from studies carried out over several decades that microtubules have a role in the translocation of cytoplasmic granules to the plasma membrane of activated neutrophils and to phagosomes within these cells (Bessis and Loquin, 1950; Malawista and Bensch, 1967; Malawista, 1971; Hoffstein et al., 1976; Hoffstein et al., 1977). Moreover, studies of vesicle movement in other types of cells have emphasized the importance of microtubules in organizing and facilitating the translocation of cytoplasmic organelles (Allen et al., 1985; Hollenbeck and Swanson, 1990; Schnapp et al., 1985; Swanson et al., 1987; Tooze and Burke, 1987). Recently, we have shown that cytoplasmic granules interact with microtubules in human neutrophils in a regulated, stimulus-responsive manner and that these interactions involve microtubule-associated ATPases as shown in other types of cells (Rothwell et al., 1989).

It is well known that tubulin, the major component of microtubules, can exist in multiple isoforms in the same cell through the expression of a variety of different tubulin genes and posttranslational modifications of the protein (Cleveland and Sullivan, 1985). It has been suggested that each isoform may subserve distinct functions (Fulton and Simpson, 1976; Stephens, 1978). However, various studies have failed to provide definite support for this hypothesis (Lewis et al., 1987; Lopata and Cleveland, 1987). Nonetheless, tubulin isoforms have not been examined rigorously with respect to their ability to support organelle-microtubule interactions. Hence the possibility remains that post-translational modifications of tubulin, e.g., the addition of acetyl (L'Hernault and Rosenbaum, 1983), tyrosine

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(Raybin and Flavin, 1977b) and glutamyl (Edde et al., 1990) residues to the α -tubulin subunit or the phosphorylation of β -tubulin (Gard and Kirschner, 1985), could affect cellular functions such as organelle translocation.

In neutrophils, granule mobilization and degranulation are highly regulated events that occur explosively when the cells are activated by phlogistic or phagocytic stimuli (Rothwell et al., 1989; Wright et al., 1977; Wright and Gallin, 1979; Wright and Malawista, 1973). Studies described in this report were directed at defining biochemical modifications to the microtubule network of stimulated neutrophils that might affect stimulus-responsive interactions between granules and microtubules. We focused in particular on the enzymatic addition of tyrosine to the carboxy terminus of the α -subunit of tubulin (tubulin tyrosination; also termed tubulin tyrosinolation). It has been reported previously by Nath and Gallin (1982) that a posttranslational tyrosination of the α -subunit of tubulin occurs in neutrophils following activation in these cells by the chemotactic peptide fMLF. Our present studies demonstrate that the levels of tyrosinated tubulin in neutrophils undergo large variations in activated cells with an initial decline in the amount of tyrosinated tubulin during the first minute following stimulation with fMLF followed by a rapid increase in tyrosinated tubulin to levels that equal or exceed those observed in resting cells. In addition, we show that the degree of tubulin tyrosination in microtubules affects the binding of isolated neutrophil granules to microtubules *in vitro*.

MATERIALS AND METHODS

Isolation of human neutrophils

Neutrophils were isolated from the venous blood of healthy volunteers by centrifugation through Ficoll-Paque (Pharmacia Inc., Piscataway, NJ), followed by dextran sedimentation and the removal of residual red blood cells by hypotonic lysis as described previously (Boyum, 1968; Wright and Gallin, 1979). These neutrophil isolates (>96% pure) were resuspended in Hanks' balanced salt solution without calcium or magnesium.

Isolation of neutrophil granules

Neutrophils were suspended in a Pipes buffer (10 mM Pipes, pH 6.9, 100 mM KCl, 3 mM NaCl, 1 mM phenylmethyl sulfonylfluoride (PMSF), 0.1 mM leupeptin, 0.1 μ g/ml aprotinin (Taylor et al., 1973)) containing 1 mM ATP. The cells were either sonicated 3 times for 10 sec or subjected to 400 psi nitrogen at 4°C in a Kontes mini-bomb (Kontes Glass Co., Vineland, NJ) to disrupt the cells by nitrogen cavitation (Borregarrd et al., 1983). Cell sonicates or cavitates were then centrifuged at 1,000g for 10 min to remove residual intact cells and nuclei (Rothwell et al., 1989). Cavitates from human neutrophils ($2-5 \times 10^7$ cells), prepared as above, were applied to a Sepharose 6B (Pharmacia-LKB Biotechnology) column (2×25 cm) and eluted with 100 mM Pipes buffer containing 1 mM MgSO_4 and 1 mM EGTA (PEM). A mixed granule population eluted with the void volume ahead of the soluble cytosolic proteins.

Preparation of microtubule protein and microtubules

Tubulin from rat brain tissue was isolated by the method of Dentler et al. (Dentler et al., 1975) in 0.1 M Pipes buffer containing 1 mM MgCl_2 , 2 mM EGTA, 1 mM GTP, and 4 M glycerol. Tubulin was purified free of microtubule-associated proteins by ion exchange chromatography using Whatman P 11 phosphocellulose (PC) (Roobol et al., 1980) and processed through a cycle of microtubule (MT) assembly and disassembly prior to use in order to remove any inactive subunits. Unless stated otherwise, MT assembly buffer was 0.1 M Na Pipes at pH 6.94 containing 1 mM MgCl_2 , 1 mM GTP and supplemented with 5% glycerol. Purified tubulin was concentrated by polymerizing phosphocellulose-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. MT were then polymerized from this protein preparation and stabilized with 1.5 μ M taxol (gift of Dr. Ven Narayanan, Drug Synthesis and Chemistry Division, NCI, Bethesda, MD), added for 20-min incubation at 37°C to stabilize MT for granule interaction studies.

Detyrosinated tubulin was prepared from phosphocellulose-purified rat brain tubulin using carboxypeptidase A conjugated to agarose beads. The enzyme was washed three times in 0.1 mM LiCl in 100 mM Pipes and added to the tubulin at 0.5U/ml tubulin (2-3 mg/ml). The mixture was incubated at 37°C for 10 min, cooled to 5°C to depolymerize any microtubules that may have started to polymerize and centrifuged twice at 10,000g to remove the beads. The detyrosinated tubulin was polymerized as above for granule binding studies.

Preparation of Neutrophils for SDS-PAGE

Purified human neutrophils were suspended in Hanks' balanced salt solution (HBSS) containing 1.25 mM MgCl_2 and 1.25 mM CaCl_2 , 1 mM PMSF, 0.1 mM leupeptin, and 0.1 μ g/ml aprotinin were added to inhibit proteolysis. Aliquots of neutrophils (100 μ l) were stimulated by adding 1 μ l 10^{-7} M (MLF (final concentration of 10^{-7} M fMLF). At 5, 30, 60, 90, and 120 sec time points, 30 μ l boiling sample buffer (containing 0.2 M Tris, pH 7.0, 20% SDS, 5 M β -mercaptoethanol, and 20% glycerol) were added to the cells. The sample was then sonicated 2×10 sec and placed on ice. Unstimulated cell samples were processed in parallel for controls. Before electrophoresis, the samples were boiled again. At this point the samples now contained the total tubulin content of the cells derived from both the microtubules and the subunit pool in a denatured form that could be probed by immunoblotting.

Procedures for SDS-PAGE and immunoblotting

Conditions for SDS-PAGE were as previously described by Murphy and Wallis (Murphy and Wallis, 1983). Proteins were fractionated on 10% acrylamide DATD (N,N'-diallyltartardiamide) gels, pH 8.8, or on 10-15% gradient Phastsystem gels (LKB-Pharmacia, Piscataway, NJ) and transferred to nitrocellulose paper (Towbin et al., 1979). The papers were then incubated

in solutions containing antibody specific for different isotypes of tubulin. Total tubulin was assessed with either the rat monoclonal antibody, YOL 134, (Accurate Chemicals, Westbury, NY) (Kilmartin et al., 1982) used at 1/500 dilution or a mouse monoclonal antibody against β -tubulin (Calbiochem, LaJolla, CA), diluted 1/250. Tyrosinated tubulin was detected with either YL 1/2 (Accurate Chemicals, Westbury, NY) (Kilmartin et al., 1982), diluted 1/500 or an antibody (TYR antibody) directed against the terminal sequence of the tyrosinated α -tubulin subunit (Gundersen et al., 1984) at 1/7,000 dilution. Detyrosinated tubulin was detected using an antibody (GLU antibody) raised against the terminal sequence lacking the final tyrosine residue at a dilution of 1/5,000 (Gundersen et al., 1984) (both anti-peptide antibodies were gifts of Dr. J.C. Bulinski, Columbia University, NY). The nitrocellulose papers were washed in Tris-buffered saline with 0.05% Tween-20 (TTBS) and incubated with alkaline phosphatase conjugated goat antirabbit immunoglobulin antibody, diluted 1/250. Antibody-antigen complexes were visualized after washing with TTBS by the addition of 100 mM Tris, pH 9.5 containing 100 mM NaCl, 5 mM $MgCl_2$, and 6.6 μ l of a stock solution of 50 mg/ml nitro blue tetrazolium in 50% dimethyl formamide and 3.3 μ l of a stock solution of 50 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate in 100% dimethyl formamide per ml of reaction buffer.

Tubulin bands from the immunoblots were quantified either by laser densitometry (Pharmacia-LKB Biotechnology, Piscataway, NJ) of blots or by analysis of images scanned into an Apple Macintosh IIcx computer with a Hewlett-Packard Scanjet IIc and quantified with the NIH Image (V. 1.44) software package. Values were plotted as the percent of tubulin levels in unstimulated control cells.

Electron microscopy

In order to quantify the frequency of granule-MT complexes in neutrophil cavities, samples were examined in a Zeiss EM109 (Carl Zeiss, Inc., Thornwood, NY) electron microscope and measurements of MT-attached granules per MT length were made. Samples containing non-taxol stabilized MT were diluted into 1% glutaraldehyde solution to fix MT before adsorption to carbon-coated parlodian grids. Samples containing taxol-stabilized MT were applied directly to the grids, and the grids were then stained with 2% uranyl acetate and examined by electron microscopy.

In order to assess the effect of tubulin isoform on granule-microtubule interactions, we used an *in vitro* granule-microtubule binding assay (Rothwell et al., 1989). Isolates of granules were prepared from neutrophils disrupted by nitrogen cavitation (Borregarrd et al., 1983) and were incubated with rat brain microtubules polymerized from carboxypeptidase A-treated (detyrosinated) tubulin or native (untreated) tubulin. The number of granules interacting with microtubules was then determined by electron microscopic examination and expressed quantitatively as the number of granules/micrometer of microtubule length or as the percentage of granules attached to microtubules per total number of granules per EM field.

All MT and granules in selected grid squares were measured until a minimum of 400 μ m polymer length (typically 25–35 MT for each experimental point) were examined to quantify granule-MT associations *in vitro* and 100 μ m polymer length (typically 35–46 MT) to quantify granule-MT associations formed *in vivo*.

Specific labeling of tyrosinated or detyrosinated tubulin was accomplished using polyclonal antibodies (GLU or TYR) to identify tubulin isoform composition of the MTs recovered from resting or stimulated neutrophils. Following glutaraldehyde fixation as described above, samples were incubated with the primary antibodies for 1 hr. This was followed by a one hour incubation with Protein A-gold and negative staining with 1.0% uranyl acetate to permit identification of cellular components (Rothwell et al., 1986).

Immunofluorescence microscopy

Cells were prepared for examination by immunofluorescence microscopy to show the changes in the microtubule network that occurs upon activation of the neutrophils. To avoid preactivation of the cells by contact with glass slides, stimulation and fixation of the cells for antibody labeling was performed on cells in suspension. The cells, at a concentration of 5 million/ml in HBSS without Ca/Mg, were warmed at 37°C for 45 min in a shaking water bath. fMLF (10 μ l aliquots at 10^{-6} M) was added to 1 ml samples (1/100 dilution) and incubated for 1 min. The cells were placed in Osborne stabilization buffer [100 mM Pipes, 1 mM EGTA, 5 mM $MgCl_2$, 4% PEG 6000, 2.5 mM GTP with 0.5% Triton for 2 min (Osborne and Weber, 1976)]. The cells were fixed for 10 min after the addition of formaldehyde to 2% and glutaraldehyde to 0.1% (final concentrations) and then centrifuged in a Cytospin (Shandon Scientific, Astmoor, England) onto glass slides. The samples were dehydrated in 20°C MeOH for 6 min and 20°C Acetone for 1 min. Following rehydration in PBS for 5 min, the slides were incubated with the following antibodies: YL 1/2 (dilution of 1/2), rhodamine-anti-rat antibody (5 μ g/ml, Sigma Chemical Co., St. Louis, MO), GLU tubulin AB (dilution of 1/40) and fluorescein-antirabbit antibody (5 μ g/ml, Boehringer Mannheim, Indianapolis, IN), for 1 hr at 37°C with 3 washes of PBS between each incubation. Before viewing, coverslips were mounted on the samples using Vectashield (Vector Laboratories, Burlingame, CA) to diminish photobleaching.

RESULTS

Levels of tyrosinated vs. detyrosinated tubulin in neutrophils

Activation of human neutrophils with the chemotactic peptide fMLF has been shown to be associated with increased tubulin tyrosination as measured by the incorporation of 14 C-tyrosine into α -tubulin subunits of neutrophil microtubules through the specific action of tubulin ligase (Nath et al., 1982; Nath and Gallin, 1986). Our initial studies were directed at determining the relative levels of tyrosinated vs. detyrosinated tubulin in both resting and activated neutrophils, since the previously reported finding of increased 14 C-tyrosine incorporation into tubulin in activated neutrophils (Nath et al., 1982) does not, by itself, constitute

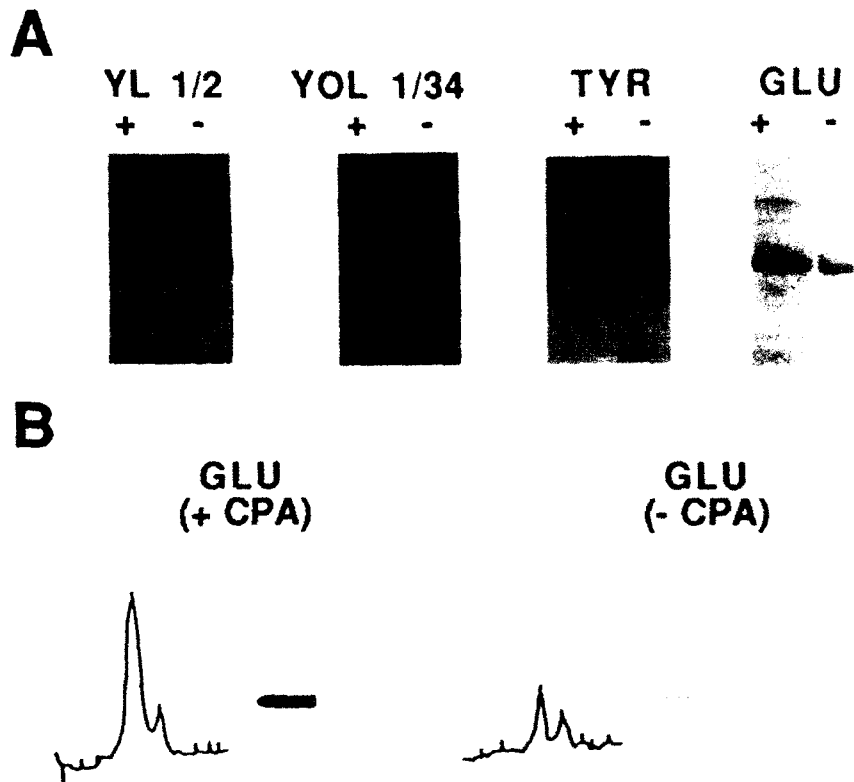


Fig. 1. Illustration of the specificities of anti-tubulin antibodies used in this study. **A:** Phosphocellulose-purified rat brain tubulin, treated with carboxypeptidase A (+) or untreated (-), was electrophoresed on 5–15% acrylamide gradient gels, transferred to nitrocellulose and immunoblotted with the following antibodies: YL 1/2 (specific for tyrosinated tubulin), YOL 1/34 (specific for total tubulin), TYR (specific

for tyrosinated tubulin), and GLU (specific for detyrosinated tubulin). The figure shows the immunoblots with the tubulin form recognized by each antibody. **B:** Immunoblots of neutrophil extracts with (+) or without (-) carboxypeptidase treatment. Densitometer scans of the tubulin band are beside each corresponding blot.

clear evidence that the relative levels of tyrosinated and detyrosinated tubulin are changed in these cells with activation. This phenomenon could reflect simply an increase in the turnover of tubulin isoforms as opposed to a change in the absolute amounts of tyrosinated tubulin.

Quantitative estimates of detyrosinated vs. tyrosinated tubulin present in resting neutrophils were made by immunoblotting neutrophil extracts with anti-tubulin antibodies that recognize different antigenic epitopes of tubulin. The reactivities of anti-tubulin antibodies used in these studies are illustrated in Figure 1. Both the GLU (Fig. 1B) and the TYR (Fig. 2) antibodies recognize a lower molecular weight band in samples prepared from neutrophil extracts. Immunoblots were performed on neutrophil extracts that had either been treated with carboxypeptidase A, to remove the terminal tyrosine from tyrosinated tubulin subunits in the extracts, or left untreated. The tubulin present in untreated extracts represented a mixture of tyrosinated and detyrosinated tubulin, while almost all the tubulin in treated extracts was detyrosinated. As a consequence of carboxypeptidase treatment the amount of tubulin in extracts that could be recognized by the GLU antibody, which binds specifically to the GEEEGEE carboxy terminal sequence of detyrosinated α -tubulin

(Gundersen et al., 1984), was increased, especially in neutrophil extracts. In contrast, the amount of tubulin that could be recognized by antibodies specific for tyrosinated tubulin subunits was diminished. Immunoblots showing increases in detyrosinated tubulin in enzyme-treated extracts allowed us to estimate the fraction of tubulin subunits that possessed a terminal tyrosine residue accessible to enzymatic cleavage by carboxypeptidase (illustrated in Figure 1B), while immunoblots using an antitubulin antibody that recognizes only tyrosinated tubulin (TYR antibody) (Gundersen et al., 1984) allowed us to determine the extent to which tubulin in the extracts was detyrosinated by carboxypeptidase treatment. Conditions of enzyme treatment which eliminated all detectable tyrosinated tubulin in preparations of purified rat brain tubulin were found to detyrosinate approximately 75% of the tyrosinated tubulin present in the human neutrophil extracts. When estimates of the ratio of tyrosinated/detyrosinated tubulin were then calculated from densitometer scans of the GLU immunoblots after correction for the percentage of tyrosinated tubulin that remained after carboxypeptidase treatment, we found that the percentage of total tubulin in resting human neutrophils that was tyrosinated was on average about 70% compared to 46% of the tubulin in rat brain extracts.

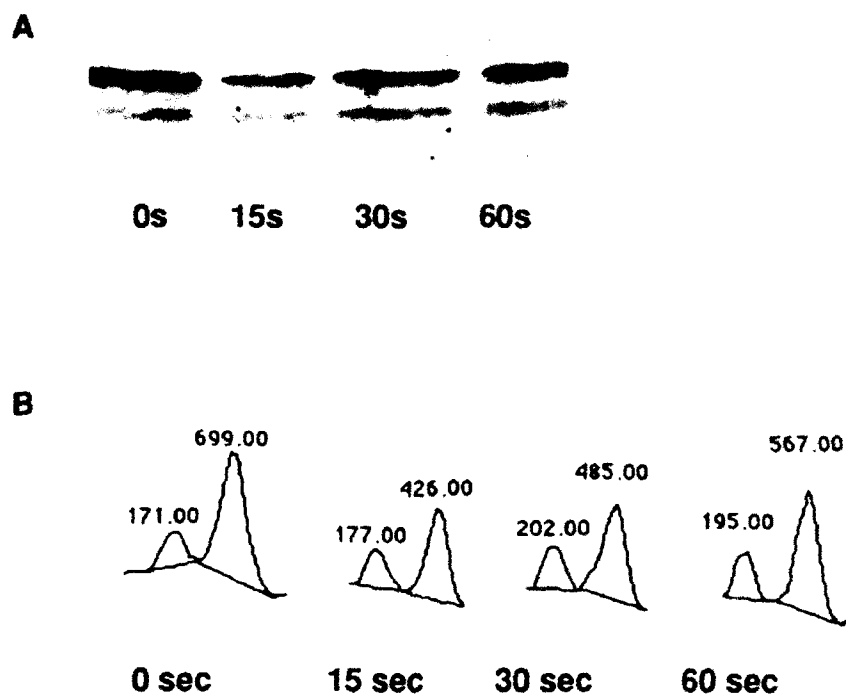


Fig. 2. Changes in the levels of tyrosinated tubulin in neutrophils stimulated with fMLF. Neutrophils were stimulated with 10^{-7} M fMLF for 5, 30, 60, 90 and 120 sec, and then sonicated and prepared for SDS-PAGE. Following electrophoresis, the samples were transferred to nitrocellulose paper and blotted with the TYR antibody which recognizes tyrosinated tubulin (A). Tubulin bands were quantified using the computer software NIH Image on scanned images (B).

Changes in the levels of tyrosinated and detyrosinated tubulin in human neutrophils following activation

In order to determine whether the absolute amounts of tyrosinated and detyrosinated tubulin in human neutrophils change following activation, we used the immunoblot approach to measure the levels of these tubulin isoforms in neutrophils at various times following activation by fMLF. For these studies, we used not only the GLU and TYR antibodies, but also the rat monoclonal antibodies, YL1/2, which recognizes the tyrosinated form of tubulin, and YOL 1/34 (Breitling and Little, 1986; Kilmartin et al., 1982), which recognizes both tyrosinated and detyrosinated tubulin subunits. As shown in Figure 1, YL 1/2 failed to detect carboxypeptidase-treated, detyrosinated tubulin, while YOL 1/34 detected both tyrosinated and detyrosinated forms of the protein.

Changes in the levels of tyrosinated tubulin in neutrophils following fMLF stimulation, as detected by immunoblotting with either the TYR or the YL1/2 antibody, are illustrated in Figure 2A,B (TYR antibody) and are shown quantitatively in Figure 3 (YL1/2 antibody). As is illustrated in these figures, the levels of tyrosinated tubulin declined rapidly during the first 15 sec following stimulation, but returned to control levels within 60–90 sec and, in most cases, exceeded these levels by 120 sec. In contrast, the total amount of tubulin, as determined by blotting with the antitubulin antibody YOL 1/34, remained relatively unchanged (Fig. 3). In separate, but comparable studies using the GLU antibody (illustrated in Figure 4), we found that the

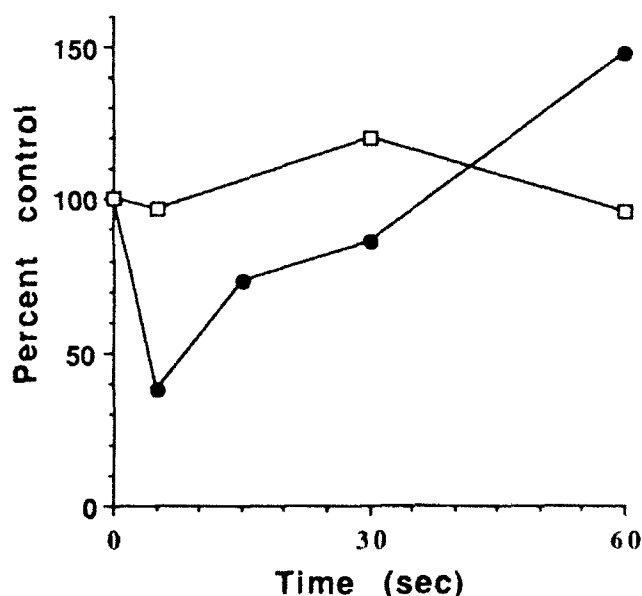


Fig. 3. Changes in the levels of tyrosinated and total tubulin in neutrophils stimulated with fMLF. Neutrophils were stimulated by 10^{-7} M fMLF and prepared for immunoblots as in Figure 2 using YL 1/2 (tyrosinated tubulin specific) and YOL 1/34 (total tubulin) antibodies. Tubulin bands were quantified by laser densitometry. Results of one representative experiment out of five replicate studies are shown. (●), tyrosinated tubulin; (□), total tubulin.

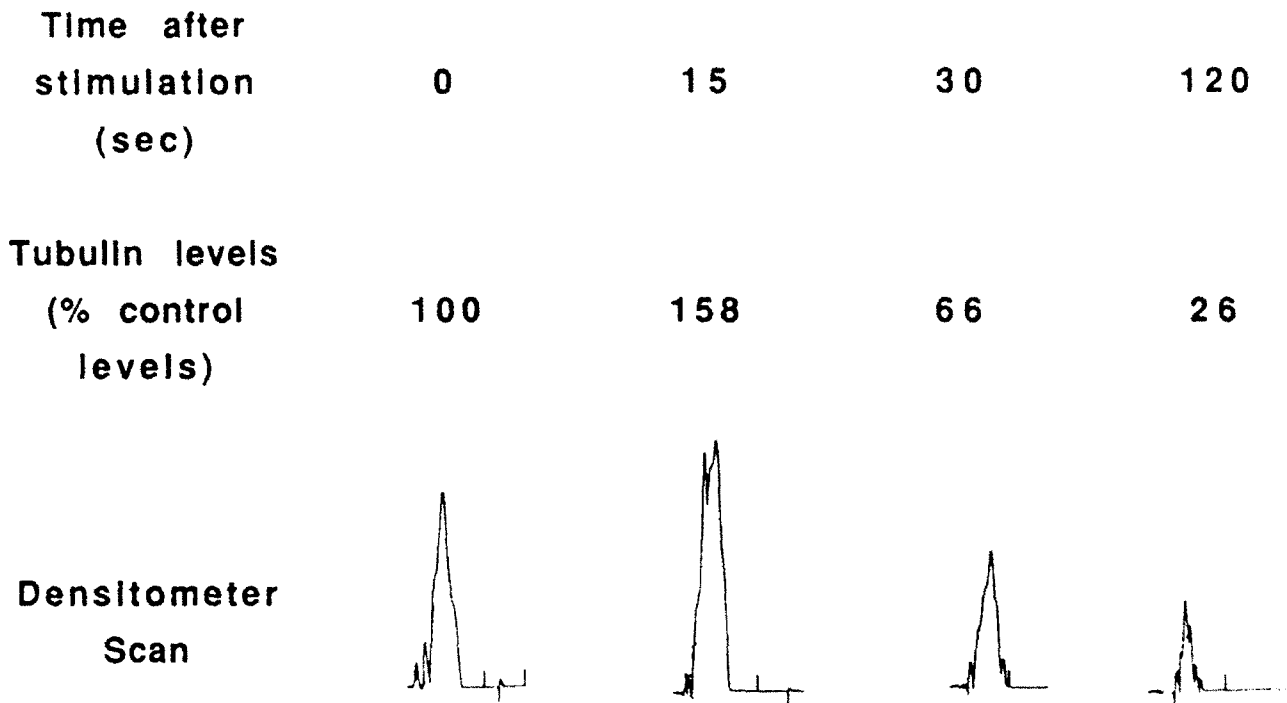


Fig. 4. Changes in the levels of deetyrosinated tubulin in human neutrophils following fMLF stimulation. Human neutrophils were stimulated with 10^{-7} M fMLF and at the time points indicated the cells were disrupted by sonication and prepared for SDS-PAGE. Levels of deetyrosinated tubulin were determined by immunoblotting with GLU antitubulin antibody and quantified by laser densitometry.

levels of deetyrosinated tubulin detected by this antibody increased rapidly after stimulation with fMLF and then declined with the same kinetics that were observed for the rapid, inverse changes in tyrosinated tubulin levels shown in Figures 2 and 3.

Changes in the levels of tyrosinated tubulin incorporated into neutrophil microtubules could also be detected using immunofluorescence microscopy. Neutrophils in suspension were stimulated with fMLF and then treated with formaldehyde/glutaraldehyde fixative to avoid activation through contact of the cells with the glass coverslip. While examination of non-stimulated neutrophils double-labeled with antibodies against the two tubulin isoforms revealed distinct microtubules arrays labeled with the YL1/2 antibody (Fig. 5A), microtubules could not be observed labeled with the GLU antibody (Fig. 5B). However, in cells fixed 1 min after the addition of fMLF, microtubules labeled with the GLU antibody were discernible (Fig. 5D). At the same time, microtubules labeled with YL1/2 antibody were still observed (Fig. 5C). In contrast, cells allowed to adhere to the coverslips prior to fixation and staining showed clear microtubule networks labeled with the YL1/2 antibody (Fig. 5E). No GLU-labeled microtubules were observed in adherent cells, even at high antibody concentrations (1:50 dilution of serum).

The distribution of tubulin isoforms in microtubules recovered from resting and activated neutrophils

Given the substantial changes in the relative amounts of tyrosinated and deetyrosinated tubulin that

were observed in neutrophils following activation, we questioned whether these changes reflected the appearance and disappearance of microtubules composed entirely of one or the other tubulin isoform, or whether these changes reflected the generation of isoform-specific domains within intact microtubules, as has been observed in the axons of sympathetic neurons by Baas and Black (1990). When resting and activated neutrophils were disrupted by nitrogen cavitation and the microtubules in the cavitates were then labeled with immunogold conjugates containing the GLU and TYR tubulin antibodies and examined by electron microscopy, we found no evidence of either microdomains of different tubulin isoforms or a mixed population of microtubules composed solely of a single tubulin type (Fig. 6).

Although no isoform-specific microtubules or microtubule domains were observed in resting or activated neutrophils, we did observe changes in the degree of uniformly distributed labeling by the two antibodies at different time points following fMLF stimulation of the cells, which were consistent with the results of immunoblot studies described above. The amount of tyrosinated and deetyrosinated tubulin in microtubules recovered from these cells was estimated by counting immunogold particles micrometer microtubule length. The ratio of tyrosinated/deetyrosinated tubulin labeling decreased from a value of 1.14 in resting cells to a value of 1.0 at 30 sec after fMLF stimulation and then recovered to a value of 1.48 at 120 sec after stimulation. The magnitude of tubulin isoform shifts measured by this approach was not as great as that detected with immu-

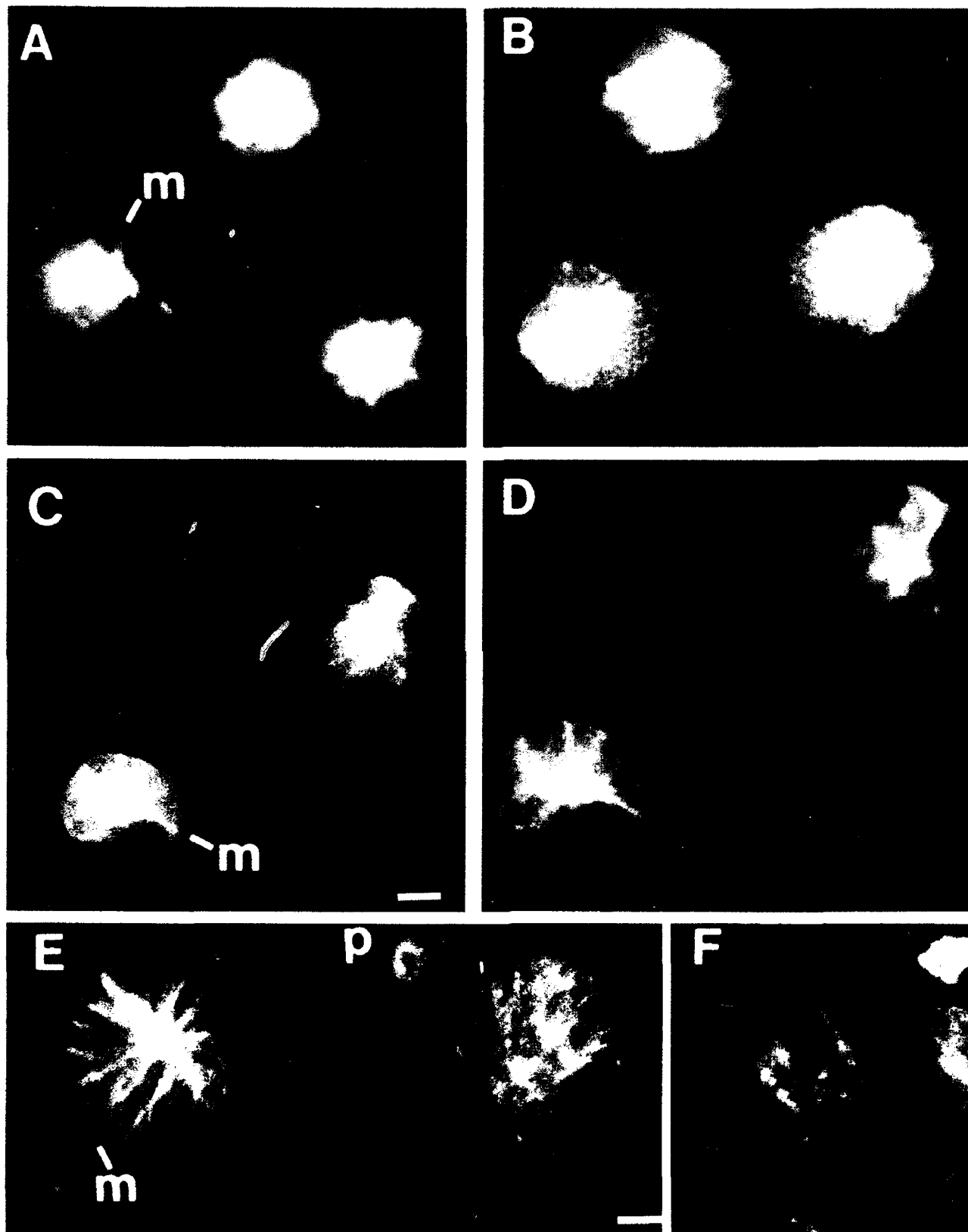


Fig. 5. Changes in the tubulin isoform content of neutrophil microtubules as detected by immunofluorescence microscopy. Non-stimulated cells (A,B,E,F) and MLF-stimulated cells (C,D) were prepared for immunofluorescence microscopy and double-labeled with YL12 and GLU anti-tubulin antibodies. A,C,E: YL12 antibody labeling. B,D,F: Microtubules labeled with GLU antibody. Cells in A,B,C,D were pre-

pared for immunofluorescence microscopy in solution, while the cells in E and F were allowed to spread on glass coverslips before fixation. Inset in F shows a platelet, taken from the same preparation as the neutrophil in E, with microtubules well labeled with the GLU antibody. m, microtubule; p, platelet. Bar: 5 μ m.



Fig. 6. Microtubules recovered from neutrophils stimulated with 10^{-7} M FMLF show no evidence of tubulin isotype domains. After stimulation, neutrophils were sonicated and diluted into 1% glutaraldehyde. Samples were incubated with TYR (A,B) or GLU (C) antibodies, followed by protein A-gold incubation and then stained with uranyl acetate negative. Bar: 90 nm.

noblots of neutrophil extracts and this difference likely reflects saturation of labeling at relatively low concentrations of one tubulin isoform or the other.

Effects of changes in levels of tyrosinated tubulin on granule-microtubule interactions

To investigate the effects of changes in the proportion of tyrosinated to detyrosinated tubulin on neutrophil granule-microtubule interactions, we used an *in vitro* granule-microtubule binding assay which we have reported previously (Rothwell et al., 1989). Isolates of granules were prepared from neutrophils disrupted by nitrogen cavitation (Borregarrd et al., 1983) and were incubated with rat brain microtubules polymerized from carboxypeptidase A-treated (detyrosinated) tubulin or native (untreated) tubulin. The number of granules interacting with microtubules was then determined by electron microscopic examination (illustrated in Fig. 7) and expressed quantitatively as the number of granules/micrometer of microtubule length or as the percentage of granules attached to microtubules per total number of granules per EM field. As discussed above, untreated rat brain tubulin obtained by cycles of assembly and phosphocellulose exchange chromatography contains a mixture of tyrosinated and detyrosinated tubulin in approximately equal proportions. When granule-microtubule interactions were mea-

sured using microtubules that contained a mixture of both tyrosinated and detyrosinated tubulin and then compared with measurements of such interactions with microtubules that contained no detectable tyrosinated tubulin, we found that significantly higher numbers of granules bound to microtubules assembled from completely detyrosinated tubulin than to microtubules that contained tyrosinated tubulin: 9.2 vs. 6.2 granules bound per 100 μ m of microtubule polymer (Table 1) or 59 vs. 44% of granules EM field bound to microtubules (mean values from 5 separate experiments).

DISCUSSION

In studies described in this report we have investigated changes in the biochemical composition of the microtubule network associated with tubulin tyrosination and detyrosination, and we have explored the possibility that changes in the relative proportions of tubulin isoforms in microtubules might modulate the interactions of granules with microtubules. Evidence that neutrophil activation is associated with stimulated tubulin tyrosination was first reported by Nath et al. (1982). Using an immunoblot approach we first determined the proportion of tyrosinated and detyrosinated tubulin in neutrophils and found that the majority of tubulin in resting neutrophils possesses a tyrosine at the carboxy terminus of the α -subunit. The tubulin α -subunit is normally synthesized with a tyrosine on the C-terminus (Lewis et al., 1985; Pratt and Cleveland, 1988; Pratt et al., 1987); however, this tyrosine can be removed by the activity of tubulin carboxypeptidase, an exopeptidase which works preferentially on the microtubule polymer (Beltramo et al., 1987; Beltramo et al., 1989). Tyrosine can then be added back through activity of tubulin tyrosine ligase, an enzyme that works preferentially on monomeric tubulin subunits (Barra et al., 1987; Beltramo et al., 1987, 1989; Raybin and Flavin, 1977a). Newly polymerized microtubules usually have a high proportion of tyrosinated tubulin (Webster et al., 1987; Wehland and Weber, 1987), and this can either be due to the incorporation of newly synthesized tubulin subunits or because pre-existing subunits were tyrosinated by the ligase before polymerization. Conversely, as microtubules age within an individual cell, the proportion of detyrosinated tubulin may increase due to the activity of the carboxypeptidase (Warn et al., 1990; Webster et al., 1990).

Levels of tyrosinated tubulin vary widely in different cell types. In our studies, we found that 60–80% of the total tubulin in resting neutrophils is in the tyrosinated form. Chapin and Bulinski have shown that greater than 80% of the tubulin found in Hela cells is tyrosinated (Chapin and Bulinski, 1991). Chinese hamster ovary cells have also been shown to have relatively high levels of tyrosinated tubulin unless the cells are treated with the drug taxol, a microtubule-stabilizing agent (Schiff et al., 1979; Schiff and Horowitz, 1980), to form stable microtubules that presumably can then be acted upon by tubulin carboxypeptidase (Wehland and Weber, 1987). On the other hand, more differentiated, nondividing cells, such as neurons and L6 muscle cells with stable microtubule arrays, have been found to have relatively high proportions of detyrosinated tubu-

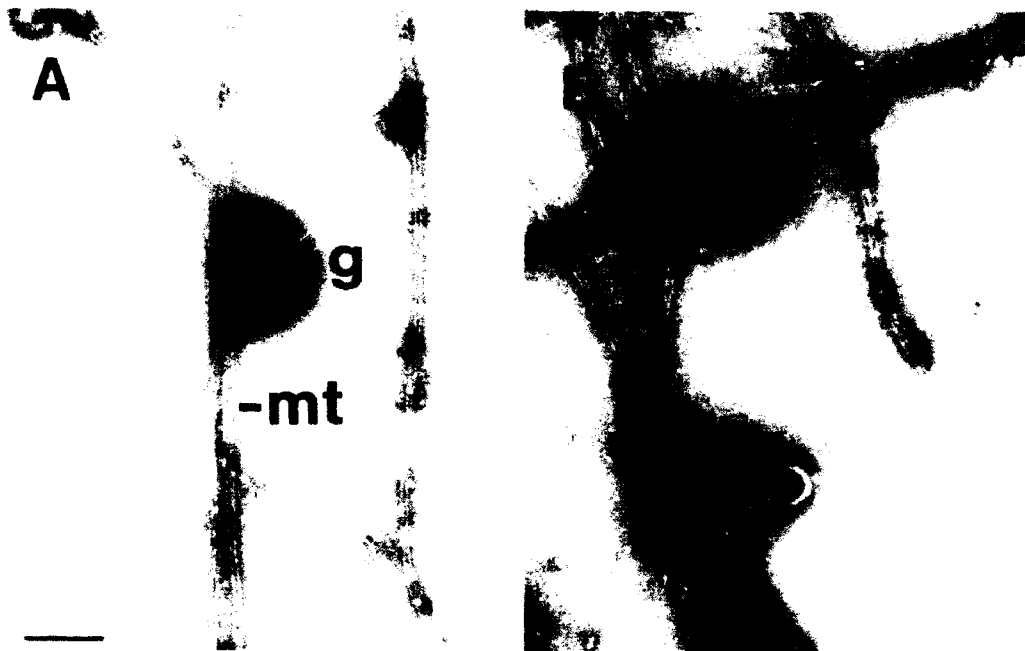


Fig. 7. Granule-microtubule interactions are increased in the presence of microtubules containing detyrosinated tubulin. Microtubules polymerized from untreated PC-purified rat brain tubulin (A) or carboxypeptidase-treated tubulin (B) were incubated with neutrophil granule preparations. Granule-microtubule interactions were then assessed by electron microscopy as described in Materials and Methods. mt, microtubules; g, granules. Bar: 0.25 μ m.

TABLE 1. Effects of tubulin tyrosination on microtubule-granule interactions.¹

Experiment	Granules 100 μ m mt tyr tubulin ²	Granules 100 μ m mt detyr tubulin ²
1	3.5	9.7
2	8.0	9.9
3	7.4	9.8
4	5.4	9.1
5	6.9	7.6
Mean $\pm$ SEM	6.2 $\pm$ 1.0*	9.2 $\pm$ 0.4

¹Neutrophil cavities were incubated with microtubules polymerized from rat PC tubulin and scored for granule-microtubule interactions by examination in the EM.

²Rat PC tubulin was treated with carboxypeptidase A to remove the terminal tyrosine of α -tubulin. (tyr, tubulin pool still containing tyrosinated tubulin; detyr, tubulin treated to remove tyrosine)

*Significantly different from detyrosinated samples; $P < 0.05$.

lin (Gundersen et al., 1989). Moreover, increased levels of detyrosinated tubulin coincident with terminal cellular differentiation have been reported by Warn et al. in their studies of the developing *Drosophila* embryo (Warn et al., 1990).

Our finding that human neutrophils possess high levels of tyrosinated tubulin contrasts with the general finding of an increased proportion of detyrosinated tubulin in differentiated, nondividing cells. However, the neutrophil has a highly dynamic microtubule network that rapidly responds to extracellular stimuli with increases in polymer number and mass (Anderson et al., 1982). Moreover, the neutrophil is a motile cell which undergoes extreme shape changes in association with rapid changes in the microtubule network and other cytoskeletal elements.

In our studies we found the stimulus-responsive tubulin detyrosination/tyrosination activity induced by the chemotactic peptide fMLF to be more rapid and complex than was apparent in the earlier studies of Nath et al. (1982). Following stimulation of neutrophils with fMLF, the level of tyrosinated tubulin declined rapidly to below 50% of initial levels within 15–30 sec. This decline in tyrosinated tubulin was then reversed by a subsequent rise in the level of tyrosinated tubulin that attained or exceeded the pre-stimulation level by 2 min following activation. These changes in levels of tyrosinated tubulin were found to occur so rapidly that we questioned whether the tubulin tyrosine ligase was capable of retyrosinating tubulin subunits so quickly. However, calculations, based on a turnover number for the tubulin ligase of 10 tubulin subunits/sec (Raybin and Flavin, 1977a), 3,700 molecules of ligase/cell (Webster et al., 1987), and an estimate of 40 microtubules of 10- μ m length (6.7×10^5 dimers) per neutrophil (Schliwa et al., 1982) indicated that the retyrosination could be accomplished in as little as 18 sec. However, there are factors other than the efficiency and turnover rate of tubulin ligase to be considered. Since the preferred substrate for the ligase is the tubulin subunit rather than the tubulin dimer incorporated into a microtubule, this suggests that the neutrophil microtubules must be detyrosinated by the carboxypeptidase and then depolymerized to subunits before retyrosination occurs. It was first thought that polymers formed from the detyrosinated isoform were more stable than microtubules polymerized from tyrosinated tubulin (Webster et al., 1987); therefore, these microtubules would depolymerize more slowly than microtubules composed of tyrosi-

nated tubulin. This would insert a slow, rate-limiting step at a critical point in the reaction pathway. However, more recent studies by Webster et al. (1990) have clarified these observations and have shown that there is no significant difference in stability between microtubules composed of de-tyrosinated and tyrosinated tubulin as assessed by immunofluorescence microscopy. If the two tubulin isoforms are approximately equivalent in their depolymerization rates, then the rates of rapid shortening of microtubules (12–17 $\mu\text{m}/\text{min}$) observed by Cassimeri in monocytes (Cassimeri, 1986) and in newt lung cells (Cassimeri, 1988) would be consistent with the time scale for the events observed in our experiments although microtubule depolymerization could still be the rate-limiting step in the retyrosination process. Certainly, these studies tell that the neutrophil microtubule network is extremely dynamic and sensitive to the activation state of these cells.

This marked change in the tubulin isoform content of neutrophils at a time when increased microtubule polymerization is known to occur (Anderson et al., 1982; Schliwa et al., 1982) suggested the possibility that the microtubules of activated neutrophils might segregate into populations of polymers composed mostly of de-tyrosinated or tyrosinated tubulin. However, immunoelectron microscopy showed a uniform distribution of tyrosinated tubulin in microtubules recovered from neutrophils at various times following stimulation. We did observe that there were definite changes observed in the density of immunogold labeling of microtubules recovered from resting and stimulated cells when an antibody specific for tyrosinated tubulin was used that corresponded to the shifts in tyrosinated tubulin measured by immunoblotting. However, there was no evidence of microtubules that contained only one tubulin isotype or of microdomains within polymers composed of only one tubulin type, as observed in the axons of neuronal cells (Baas and Black, 1990).

We also observed that granule-microtubule interactions are affected by changes in the microtubule composition. Interactions between neutrophil granules and reconstituted microtubules, as measured by direct electron microscopic examination, were significantly increased when microtubules were composed entirely of de-tyrosinated tubulin, as compared with microtubules containing tyrosinated tubulin. This finding is of particular interest, for in previously reported studies (Rothwell et al., 1989) we showed that fMLF stimulation induced a rapid rise in microtubule-granule associations within the cell during the first 2 min of activation, coincident with the time period which we now show to be associated with tubulin de-tyrosination in the microtubule network.

The ability of a single amino acid change at the carboxy terminus of the tubulin subunit to modify the activity of microtubule-dependent ATPases is not an unreasonable possibility since the carboxy region of tubulin subunits is exposed both in free tubulin dimers and in intact microtubule polymers and is known to contain the binding region for a number of microtubule-binding proteins (Littaur et al., 1986; Serrano et al., 1984a,b). However, contradictory studies have been reported by Paschal et al. (1989), and by Cleveland et al. (1990), and Rodionov et al. (1990) regarding the impor-

tance of this region of the tubulin molecule for kinesin and dynein binding during the enzymatic cycle of these molecular motors. Our findings of rapid changes in tubulin tyrosination/de-tyrosination in activated neutrophils, together with changes in microtubule-granule interactions that were associated with changes in the degree of tubulin tyrosination/de-tyrosination in the microtubule network, indicate that neutrophils may provide a useful experimental system with which to examine the effects of tubulin tyrosination on kinesin and dynein ATPase activation.

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