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DIFFERENCES IN SIGNAL TRANSDUCTION BETWEEN Fcγ RECEPTORS (FcγRII, FcγRIII) AND FMLP RECEPTORS IN NEUTROPHILS

Effects of Colchicine on Pertussis Toxin Sensitivity and Diacylglycerol Formation[1]

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Studies on the role of microtubule integrity in stimulus-response coupling in neutrophils have generated contradictory data. To determine the role of microtubule integrity in stimulus-response coupling elicited by two different mechanisms, i.e., engagement of the Fc receptors (FcRγII, FcRγIII) or engagement of the receptor for FMLP, we utilized colchicine (10 μM), which reduces pericentriolar microtubules to 29% of control, and compared its effect with that of nocodazole (50 μM) and lumicolchicine (10 μM). We now demonstrate that treatment of neutrophils with colchicine but not lumicolchicine, *inhibits* degranulation elicited by engagement of Fc receptors but *augments* degranulation in response to FMLP. In contrast to the ligand-specific effect of microtubule-disruption on degranulation, superoxide anion production (assembly of the NADPH oxidase) is unaffected by colchicine regardless of the ligand. To determine whether intact microtubules were required for responses elicited by ligation of FcγRII(CD32) or FcγRIII(CD16), mAb directed against these receptors were employed. Treatment of neutrophils with mAb KuFc79 directed against FcγRII(CD32) or mAb 3G8 directed against FcγRIII(CD16) inhibited degranulation of neutrophils elicited by immune complexes (IC). In contrast, removal of most of FcγRIII by phosphatidylinositol-specific phospholipase C did not significantly alter degranulation in response to IC. We conclude that degranulation elicited by IC results from ligation of both FcγRII and phosphatidylinositol-specific phospholipase C-insensitive FcγRIII. The importance of microtubule integrity on the generation of intracellular signals was also examined. Degranulation of neutrophils proceeds via pertussis toxin-sensitive and insensitive pathways; treatment of cells with colchicine did not augment the action of pertussis toxin. Stimulation of neutrophils by chemoattractants results in a biphasic increase in 1,2-*sn*-diacylglycerol; a rapid increase ("triggering") secondary to the action of a phosphatidylinositol-specific phospholipase C, and a late increase ("activation") secondary to the action of a phosphatidylcholine-specific phospholipase C. Treatment of cells with colchicine altered the production of both [^{3}H]-arachidonic acid-diacylglycerol and diacyl[^{14}C]glycerol in parallel to its effect on degranulation. These studies indicate that the requirement of intact microtubules for degranulation is ligand-specific. Furthermore, assembly of the respiratory burst oxidase does not require intact microtubules. Microtubules most likely alter the cycling of specific receptors or the generation of specific intracellular signals required for stimulus-response coupling in the course of degranulation. Intact microtubules are not uniformly required for the discharge of granule contents during exocytosis.

Fusion of lysosomes with phagosomes and the exocytosis of lysosomal contents are major components of the inflammatory response of neutrophils. Degranulation follows engagement of receptors and activation of a pertussis toxin-sensitive G_{pc},[4] which, via phospholipase C, hydrolyzes phosphatidylinositol-1,4,5-bis-phosphate to generate inositol trisphosphate and 1,2-*sn*-DG (1–3). Generation of 1,2-*sn*-DG follows biphasic kinetics ("triggering" and "activation") as determined by simultaneous measurements of [^{3}H]AA-DG and diacyl[^{14}C]glycerol (4, 5). The "early" response (triggering) is secondary to the hydrolysis of phosphatidylinositol-1,4,5-bis-phosphate, whereas in the "late" response (activation), DG is derived from another source (de novo synthesis, phosphatidylcholine). With the use of a translocatable porin, protein I of *Neisseria gonorrhoeae* we have demonstrated the activation of a PI-PLC that participates in the generation of the "late" increase in 1,2-*sn*-DG (5, 6). Additional phosphoinositides including phosphatidylinositol trisphosphate or tetrakisphosphate have also been implicated in cell activation (7). Whereas inositol trisphosphate elicits a rise in cytosolic free calcium, 1,2-*sn*-DG activates C-kinase resulting in the phosphorylation of target proteins.

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[4] Abbreviations used in this paper: G_{pc}, GTP-binding protein; MT, microtubules; IC, immune complexes; DG, diacylglycerol; O_2^-, superoxide anion; AA, arachidonic acid; PI-PLC, phosphatidylinositol-specific phospholipase C; LDH, lactate dehydrogenase; GBD, guanine nucleotide-binding proteins.

In many cell types the movement of intracellular granules is an energy-dependent event (ATP) and requires intact MT (8–12). Indeed, at least two MT-based motors have been described, kinesin (124-kDa heavy chain, 64-kDa light chain), and cytosolic dynein (MAP 1C), both with ATPase activity (13–15). These proteins direct anterograde (kinesin) and retrograde (cytosolic dynein) movement of vesicles along MT in cell-free MT preparations and on squid axonemes. Kinesin has also been identified in non-neural cells such as fibroblasts, heart, and liver cells (16–18).

Since 1969, the role of MT integrity in the activation of neutrophils has been a matter of some controversy. Most recently we demonstrated that disruption of MT inhibits not only lysosomal enzyme release, but also the full activity of the 5-lipoxygenase (19). Lysosomal granules in neutrophils are often closely apposed to MT, and centripetal movements of granules have been demonstrated both by electron microscopy and microcinematography (17, 20–22). Moreover, an ATPase associated with both MT and granules has recently been described (17). Indeed, kinesin itself has been identified by gel electrophoresis and immunoreactivity in neutrophils (17). It would therefore be expected that granules in the course of their release move by pathways regulated by MT.

Were assembled MT and associated motors to be required for degranulation, disassembly of MT would be expected to suppress secretion of lysosomal enzymes. Indeed, previous investigators have demonstrated that treatment of neutrophils with either colchicine or vinblastine inhibits degranulation elicited by IC (Fcγ receptor), zymosan-treated serum (C3b,C3bi,Fcγ), and heat-killed staphylococci (22–29). However, the importance of intact MT for degranulation has been called into question. Colchicine neither completely inhibits degranulation elicited by particulate stimuli nor inhibits degranulation triggered by Ca^{2+} ionophore (22). Furthermore, colchicine does not inhibit lysosomal fusion in macrophages (30, 31).

We now examine the importance of intact MT for activation of neutrophils (degranulation, O_2^- generation) and the generation of intracellular signals. We employ two agents that disassemble MT, colchicine (10 μM), at a dose that we have previously demonstrated to reduce the number of intact MT (14 to four pericentriolar MT/cell) (19, 32), and nocodazole (50 μM). The inactive isomer of colchicine, lumicolchicine (10 μM), is used as a control. We demonstrate that the requirement for intact MT for degranulation is ligand specific; colchicine suppressed degranulation provoked by IC (BSA anti-BSA) but augmented that in response to the chemotactic peptide FMLP. In contrast, the generation of O_2^- elicited by either ligand was unaffected by pretreatment of cells with colchicine. Degranulation provoked by IC was mediated via both FcγRII(CD32) and FcγRIII(CD16) molecules as blocking the expression of either of these receptors with mAb (KuFc79, 3G8) inhibited degranulation. However, molecules of FcγRIII(CD16) that were sensitive to cleavage by PI-PLC were not critical for degranulation, because their removal did not inhibit enzyme release. Moreover, MT participate in a pertussis toxin-sensitive pathway of activation because colchicine did not further the inhibition of degranulation demonstrated with pertussis toxin. Finally, colchicine altered the generation of the intracellular messenger 1,2-*sn*-DG; both [^{3}H]AA-DG and diacyl[^{14}C] glycerol formed in response to IC or FMLP was altered in parallel with the effect of colchicine on degranulation. We conclude that the requirement of intact MT for optimal degranulation is ligand-specific. In contrast, colchicine does not affect the generation of O_2^-. MT are not universally required for ligand-induced secretion but alter the transduction of specific intracellular signals required for this process.

MATERIALS AND METHODS

HEPES buffer (150 mM Na^+, 1.2 mM Mg^{2+}, 1.3 mM Ca^{2+}, 155 mM Cl^-, 10 mM HEPES, pH 7.43) was used for all cell suspensions. Cytochalasin B was purchased from Aldrich (Milwaukee, WI). FMLP was obtained from Vega Biochemicals (Tucson, AZ), stored as a concentrated stock solution in DMSO, and diluted into buffer before use. The final concentration of DMSO was less than 0.01%. IgG fraction rabbit anti-BSA was obtained from Cappel (West Chester, PA). [^{3}H]AA (sp. act., 94.5 Ci/mmol) was obtained from Du Pont Biomedical Products (North Billerica, MA) and [^{14}C]glycerol (sp. act., 171 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). Ficoll 400, horse ferricytochrome *c*, BSA, lipid standards, colchicine, and nocodazole were obtained from Sigma Chemical Co. (St. Louis, MO). Pertussis toxin was obtained from List Biological Laboratories, Inc. (Campbell, CA). mAb KuFc79 and 3G8 were the kind gifts of Dr. Robert P. Kimberly (The Hospital for Special Surgery, Cornell University Medical College, New York, NY), and Dr. Shaun Ruddy (Medical College of Virginia, Virginia Commonwealth University, Richmond, VA). PI-PLC (*B. thuringienses*) was the generous gift of Dr. Martin Low (College of Physicians and Surgeons of Columbia University, New York, NY).

METHODS

Preparation of cell suspension. Heparinized (10 U/ml) venous blood was obtained from healthy donors. Purified preparations of polymorphonuclear leukocytes were prepared by means of centrifugation over hypaque/Ficoll cushions followed by dextran sedimentation and hypotonic lysis of erythrocytes as described by Boyum (33). Isolated cells (95 to 98% polymorphonuclear leukocytes) were suspended in HEPES buffer, which was used for all subsequent incubations. BSA anti-BSA was prepared as described (34).

Measurement of granule enzyme release. The extracellular release of the granule-associated enzymes lysozyme and β-glucuronidase was measured as described previously (35). β-Glucuronidase, a marker of azurophil granules, was measured by incubation of cell supernatants with phenolphthalein glucuronidate as substrate. For all experiments combined, cells released a mean of 9.2 ± 0.7% of total β-glucuronidase in response to IC and 24.4 ± 1.5% in response to FMLP. Background release of 3.8 ± 0.8% of total β-glucuronidase was subtracted. Lysozyme was measured by the rate of lysis of *Micrococcus lysodeikticus*, determined by the decrease of absorbance at 450 nm. Total enzyme activities of the cell were determined simultaneously in duplicate reaction mixtures containing detergent Triton X-100 (0.2%). Background release of 7.8 ± 0.9% was subtracted. Cells released a mean of 29.7 ± 3.1% of total lysozyme in response to IC and 44.0 ± 3.1% in response to FMLP.

O_2^- generation. The generation of O_2^- by neutrophils was measured in the presence of cytochalasin B (5 μg/ml) as the superoxide dismutase-inhibitable reduction of cytochrome *c* over 5 min. Cells were incubated at 37°C for 5 min after the addition of stimulus. The quantity of O_2^- released was determined as the difference between the readings of superoxide dismutase and nonsuperoxide

dismutase samples at 550 nm (36).

DG formation. Neutrophils (75×10^6/ml) were suspended in HEPES buffer containing fatty acid-free BSA (0.1%), [^{3}H]arachidonate (3 μCi/ml), and [^{14}C]glycerol (7 μCi/ml) and incubated (30 min, 37°C) as described previously (4). Cells were then incubated (30 min, 37°C) in the absence or presence of colchicine (10 μM). Unincorporated label was removed by washing cells twice in HEPES-BSA and cells were resuspended in HEPES. Neutrophils (17×10^6/ml) were incubated (5 min, 37°C) in the presence or absence of cytochalasin B (5 μg/ml) and treated with the appropriate stimulus for various times. The reaction was terminated by the addition of 3.5 ml of chloroform/methanol (2/5, v/v). Samples were extracted by a modified Bligh and Dyer (37) technique. Organic and aqueous phases of the extracts were separated by the addition of 1 ml of chloroform and 1 ml of distilled water followed by centrifugation. The aqueous phase was discarded and chloroform phases were taken to dryness under N_2. Lipids were resuspended in 50 μl of chloroform/methanol (2/5, v/v). Samples were applied to heat-inactivated one-dimensional Silica Gel GF plates and run using the solvent system hexane/ether/acetic acid (50/50/1, v/v/v). Lipids were visualized by iodine staining, scraped off the plates, and counted in Dimiscint (National Diagnostics) in a Beckman LS-7000 scintillation counter. DG was identified in comparison with known standards of diolein, 1,2-distearoyl-racemic-glycerol, 1,2-dioleoyl-*sn*-glycerol, 1-stearoyl-2-arachidonoyl-*sn*-glycerol (approximately 97 to 99% pure). In this system, acyl-chain substitution does not alter the mobility of DG, whereas 1,3-DG runs separately from 1,2-DG.

Immunofluorescence analysis. After treatment of neutrophils with various concentrations of PI-PLC (30 min, 37°C), neutrophils (10^6) were suspended in iced HEPES buffer (0.1 ml) containing mAb at a 1:400 dilution of ascites fluid (20 min, 4°C). Cells were washed three times and resuspended in 0.1 ml of buffer containing an excess of GAM-FITC (20 min, 4°C). Cells were again washed three times and fixed with paraformaldehyde (1% w/v) and kept refrigerated before cytofluorographic analysis. Cells were analyzed with an Ortho 5OH cytofluorograph interfaced with an Ortho 2150 computer (Ortho Diagnostic Systems, Inc., Raritan, NJ) run in the linear mode.

Cell viability. The release of LDH was monitored as a measure of cell viability. Cells were incubated (37°C) for the appropriate amount of time, centrifuged at 4°C, and assayed for LDH activity by the method of Wacker (38). Results are expressed as the percent of total LDH released after treatment with Triton X-100 (0.2%).

RESULTS

The effect of MT disrupting agents on lysosomal enzyme release. To confirm previous results showing that MT-disrupting agents inhibit degranulation of neutrophils (22, 23, 25–29), we examined the effect of colchicine and nocodazole on lysosomal enzyme release after IC (200 μg/ml) or the chemoattractant FMLP (0.1 μM). Neutrophils were incubated (45 min, 37°C) in the presence or absence of colchicine (10 μM), lumicolchicine (10 μM), the inactive isomer of colchicine, or nocodazole (50 μM). These doses inhibit MT formation maximally as previously determined by ultrastructural stereology (4, 32). Cells were treated with cytochalasin B (37°C, 5 min) and subsequently with BSA anti-BSA (200 μg/ml) or FMLP (0.1 μM) for 5 min. Cells treated with IC released 10.3 ± 0.7% β-glucuronidase and 41.6 ± 2.8% lysozyme. Cells treated with FMLP released 26.3 ± 1.5% of total β-glucuronidase and 30.9 ± 6.1% of total lysozyme. Pretreatment of cells with colchicine caused a dose-dependent decrease in the release of β-glucuronidase elicited by IC with a maximal inhibition to 48.7 ± 7.6% of control (mean ± SEM, $n = 4$) (Fig. 1). As demonstrated with colchicine, nocodazole inhibited degranulation elicited by IC (71.3 ± 3% of control, $n = 6$) (Fig. 2). In contrast, lumicolchicine had no effect on the release of β-glucuronidase elicited by IC (104.4 ± 1.2% of control, $n = 4$). Thus, in accordance with previously published literature (29), inhibition of MT assembly only incompletely suppressed degranulation. In contrast, degranulation stimulated by FMLP was not inhibited by either colchicine or nocoda-

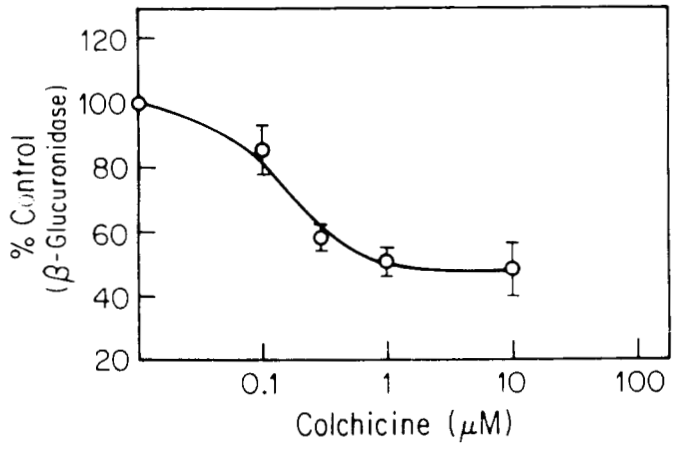

Figure 1. Dose-dependent effect of colchicine on degranulation. Neutrophils (5×10^6) were incubated with increasing concentrations of colchicine (45 min, 37°C) and stimulated by the addition of IC (200 μg/ml, 5 min). β-Glucuronidase was measured as described in *Methods*. Results are expressed as percent of the control response of 10.3 ± 0.7% of total β-glucuronidase after subtraction of background spontaneous release (5.6 ± 0.7%).

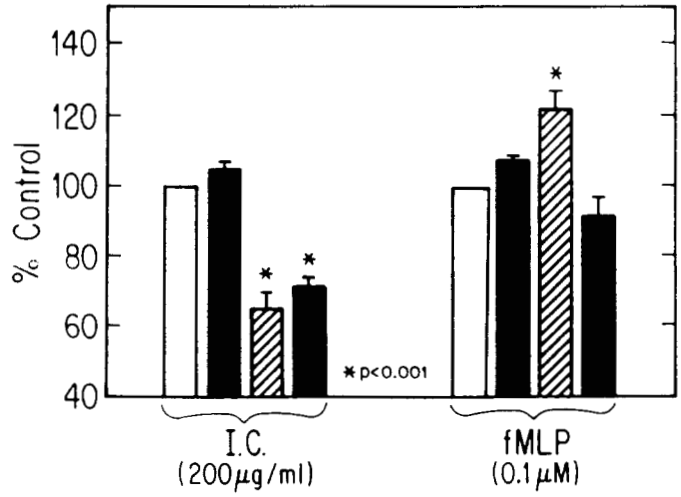

Figure 2. Effect of inhibition of MT assembly on secretion elicited by IC or FMLP. Neutrophils were preincubated with buffer (□), lumicolchicine (10 μM) (▩), colchicine (10 μM) (▨), or nocodazole (50 μM) (■), (45 min, 37°C). Cells (5×10^6) were then stimulated with either IC or FMLP (0.1 μM) (5 min, 37°C). Results are expressed as percent of control response to stimulus alone ($n \geq 8$). Cells released a mean of 10.3 ± 0.7% of total β-glucuronidase in response to IC and 26.3 ± 1.5% in response to FMLP after subtraction of background spontaneous release (3.8 ± 0.5%).

zole; colchicine augmented degranulation to 122.2 ± 5.1%, $n = 11$ ($p \leq 0.01$) of β-glucuronidase released by FMLP alone (Fig. 2).

Inhibition of MT assembly had a similar, although less marked effect on the release of lysozyme; colchicine and nocodazole decreased the release of lysozyme to 82.5 ± 4.6 and 87.4 ± 3.5% ($p \leq 0.03$) of control, respectively, after IC. In contrast, as seen with β-glucuronidase, colchicine enhanced the release of lysozyme in cells exposed to FMLP (136.0 ± 12% of control, $p \leq 0.02$). A mean of 41.6 ± 2.8% of total lysozyme was released in response to IC and 30.9 ± 6.1% in response to FMLP after subtraction of background (7.7 ± 0.9%).

Despite prolonged incubation, cells were viable as measured by the release of the cytosolic enzyme LDH (enzyme release was less than 6% of total LDH).

The effect of disrupting MT on O_2^- generation by neutrophils. The requirement of intact MT for the generation of O_2^- was also examined in neutrophils. Neither colchicine (Fig. 3) nor nocodazole (data not shown) decreased the ability of cells to generate O_2^- in response to IC or FMLP. Since the generation of O_2^- by neutrophils treated with FMLP is more susceptible to changes in ligand-receptor binding than degranulation (36), the inhibition of degranulation by colchicine was most likely not due to a decrease in ligand-receptor binding. Moreover, since the generation of O_2^- after IC was unaffected by colchicine, assembled MT are not required for the binding of IgG to its receptor.

Effect of blocking specific Fcγ receptors on degranulation. Neutrophils contain primarily two Fcγ receptors, FcγRII(CD32) and FcγRIII(CD16) (39–41). Since optimal degranulation elicited by IC required intact MT, we sought to determine whether engagement of a single class of Fcγ receptor was responsible for degranulation elicited by these IC. Neutrophils were incubated with KuFc79($IgG2_b$), a murine mAb directed against human FcγRII (42) (1:400, 30 min, 37°C), washed, and then exposed to IC in the presence of cytochalasin B. Treatment of cells with mAb alone did not elicit enzyme release above background.

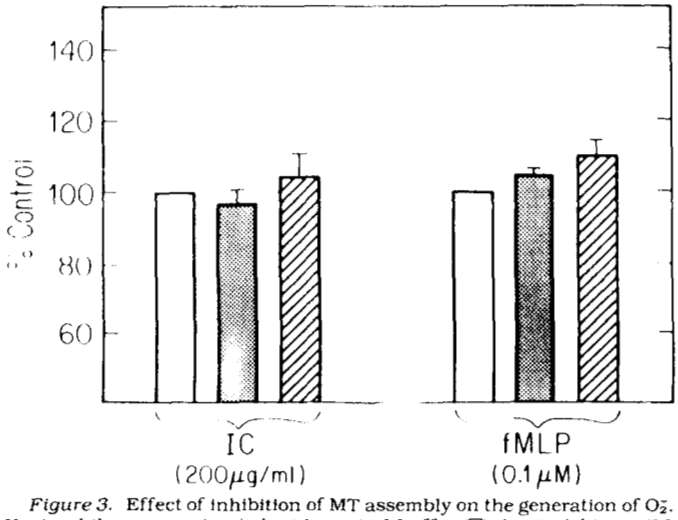

Figure 3. Effect of inhibition of MT assembly on the generation of O_2^-. Neutrophils were pretreated with control buffer (□), lumicolchicine (10 μM) (▩), or colchicine (▨), as described previously. Cells (2.5×10^6) were exposed to the appropriate stimulus (5 min, 37°C). O_2^- generation was calculated by determining the non-superoxide dismutase-inhibitable reduction of ferricytochrome *c*. Cells reduced 21.5 ± 2.5 ($n = 7$) nm of cytochrome *c* in response to IC and 29.2 ± 2.7 nm ($n = 8$) in response to FMLP.

Pretreatment of cells with mAb KuFc79 inhibited the release of β-glucuronidase elicited by IC to 39.8 ± 2.0% of control values ($n = 4$) (Table I). Similarly, pretreatment of cells with KuFc79 also inhibited the release of lysozyme elicited by IC to 21.2 ± 12.4% control ($n = 3$).

In neutrophils, FcγRIII is a phosphatidylinositol-glycan anchored molecule with two allotypes (NA1 and NA2) (43–46). The mAb 3G8 recognizes both allotypes (43). Pretreatment of neutrophils with this mAb alone did not elicit enzyme release above background. As demonstrated in Table I, pretreatment of cells with the mAb 3G8 profoundly inhibited degranulation elicited by IC; the release of β-glucuronidase was inhibited to 25.8 ± 4.5% of control ($n = 4$), and the release of lysozyme to 13.8 ± 3.6% ($n = 4$). Thus, both FcγRII and FcγRIII are involved in degranulation elicited by IC.

FcγRIII has a phosphatidylinositol-glycan anchor and is released by exogenous PI-PLC (43, 47, 48). Cells treated with 0.03 to 5 U/ml of PI-PLC and stained with FITC-conjugated GRM1 (anti-FcγRIII) demonstrated a maximal decrease in mean channel fluorescence to 36.3% of control at 0.03 to 5 U/ml. This degree of decrease in mean channel fluorescence was reached by 30 min and was consistent with previously published results (43, 47, 48). Mean channel fluorescence was not reduced in control cells stained with an isotype control (UPC10), a mAb directed against a common HLA determinant (W6/32), or a mAb directed against FcγRII molecules (CLKM5). We therefore examined whether removal of FcγRIII molecules with PI-PLC would alter enzyme release. Treatment of cells with PI-PLC alone did not increase enzyme release above background. Moreover, despite the significant removal of FcγRIII by PI-PLC, degranulation elicited by IC was not significantly reduced; after treatment with up to 5 U/ml of PI-PLC, β-glucuronidase was 91.3 ± 3.1% of control value ($n = 3$). Despite pretreatment of cells with PI-PLC, colchicine continued to decrease the release of β-glucuronidase to 60.1 ± 5.8% of control ($n = 3$). Furthermore, whereas pretreatment of cells with PI-PLC (1 U/ml) did not inhibit degranulation elicited by IC, degranulation was inhibited after subsequent exposure of cells to mAb 3G8 (Table I). We conclude that molecules of FcγRIII remaining after treatment with PI-PLC are still func-

TABLE I

Role of FcγRII(CD16) and FcγRIII(CD32) in degranulation elicited by IC[a]

Treatment	β-Glucuronidase (% control)	Lysozyme (% control)
α-FcRII + IC	39.8 ± 2.0[b]	21.1 ± 12.4[b]
MOPC 195 + IC	95.7 ± 9.9	97.8 ± 12.1
αFcRIII + IC	25.8 ± 4.5[c]	13.8 ± 3.6[c]
MOPC 21 + IC	110.8 ± 15.6	100.9 ± 10.7
PIPLC(IU) + IC	101.1 ± 8.6	89.3 ± 9.3
PIPLC + αFcRIII + IC	33.3 ± 12.1[c]	30.2 ± 13.7[b]

[a] Cells were incubated with mAb KuFc79 directed against FcγRII or with mAb 3G8 directed against FcγRIII (30 min) or appropriate isotype control (MOPC 156 or MOPC 21) as described in *Methods*. Cells were washed and subsequently stimulated with IC. β-Glucuronidase and lysozyme were measured as described in *Methods*. Results are expressed as percent control. To remove FcγRIII, cells were incubated with PI-PLC (30 min, 37°C) and subsequently stimulated with IC or pretreated with mAb 3G8 before exposure to IC. All results are expressed as percent of control response. Cells released a mean of 10.2 ± 1.4% of total β-glucuronidase after subtraction of background release of 4.3 ± 0.7% and 31.6 ± 3.8% of total lysozyme after subtraction of background release of 3.8 ± 1.5%.

[b] $p \leq 0.03$ ($n \geq 3$).

[c] $p \leq 0.007$ ($n \geq 3$).

tional, whereas FcγRIII molecules that are removed by PI-PLC are not critical for degranulation.

Effect of pertussis toxin on degranulation. Engagement of the FMLP receptor activates a G_{pc} as an early intracellular signal for degranulation (1). Pertussis toxin, via ADP-ribosylation of this GTP-binding protein, inhibits neutrophil functions such as degranulation and O_2^- generation (49–51). In contrast to FMLP, degranulation elicited by surface bound IgG or engagement of FcγRII(CD32) is incompletely inhibited by pertussis toxin (52, 53). Thus engagement of FcγRII(CD32) activates both a pertussis toxin-sensitive and -insensitive pathway. We sought to determine whether the pertussis toxin-sensitive pathway of activation required intact MT. Neutrophils were pretreated with pertussis toxin (400 ng/ml, 90 min) and stimulated with FMLP or IC. As noted by previous investigators, pertussis toxin profoundly inhibited degranulation elicited by FMLP (10^{-7} M) (18.9 ± 1.8% of control (mean ± SEM, $n = 3$, $p \leq 0.004$)) (Fig. 4). Addition of colchicine to these inactivated cells did not further the inhibition elicited by pertussis toxin (25.6 ± 1.2% control, $p \leq 0.05$ presence vs absence of colchicine ($n = 3$)). In contrast to the effect of pertussis toxin on enzyme release elicited by FMLP, that elicited by IC was only incompletely inhibited (67.5 ± 9.1% control, $n = 5$, $p \leq 0.01$). Since colchicine neither augmented nor reversed this inhibition, we suggest that colchicine affects the pertussis toxin-sensitive pathway of IC-mediated activation.

Effect of colchicine on phospholipid remodeling. Stimulation of G_{pc} in neutrophils activates phospholipase C and elicits the generation of inositol phospholipids and 1,2-*sn*-DG (50, 51, 54). We examined whether the formation of 1,2-*sn*-DG in response to IC required intact MT. Neutrophils (17×10^6/ml) were labeled with [^{3}H] arachidonate (3 μCi/ml) and [^{14}C]glycerol (7.5 μCi/ml) to equilibrium, washed, and then incubated in the absence or presence of colchicine (10 μM). IC (200 μg/ml) were added (in the presence of cytochalasin B (5 μg/ml)), and the reaction was terminated at various time points by the addition of iced chloroform/methanol. A modified Bligh-Dyer extraction was performed, and lipids were separated by TLC and identified by comparison with known standards. Spots were scraped and counted for radioactivity. Treatment of neutrophils with IC (200 μg/ml) in the presence of cytochalasin B caused a biphasic increase in [^{3}H] AA-DG with an "early" (triggering) and "late" (activation) increase (117.9 ± 7.2 and 232 ± 34.4% of control at 5 and 300 s, respectively, $n = 3$) (Fig. 5). As previously demonstrated (4), treatment of cells with FMLP (10^{-7} M) caused a biphasic increase in [^{3}H]AA-DG (112.7 ± 5.3 and 157 ± 13.7% above resting levels at 5 and 300 s, respectively) (Fig. 6*a*). Pretreatment of cells with colchicine caused a decrease in primarily the "late" [^{3}H]AA-DG formation elicited by IC (99.4 ± 4.8 and 139.5 ± 3.4% of resting levels at 5 and 300 s, respectively) (Fig. 5*a*). A similar effect was seen on diacyl[^{14}C]glycerol (Fig. 5*b*). In contrast, pretreatment of cells with colchicine caused a slight but significant increase in the "late" (300 s) rise of [^{3}H]AA-DG elicited by FMLP (10^{-7} M) (198 ± 16.8% above resting at 300 s, $n = 4$) (Fig. 6*a*). Colchicine caused a similar effect on the "late" increase of diacyl[^{14}C]glycerol formed in response to FMLP (Fig. 6*b*). The alterations in the formation of 1,2-*sn*-DG parallel the effects of colchicine on degranulation provoked by these two ligands.

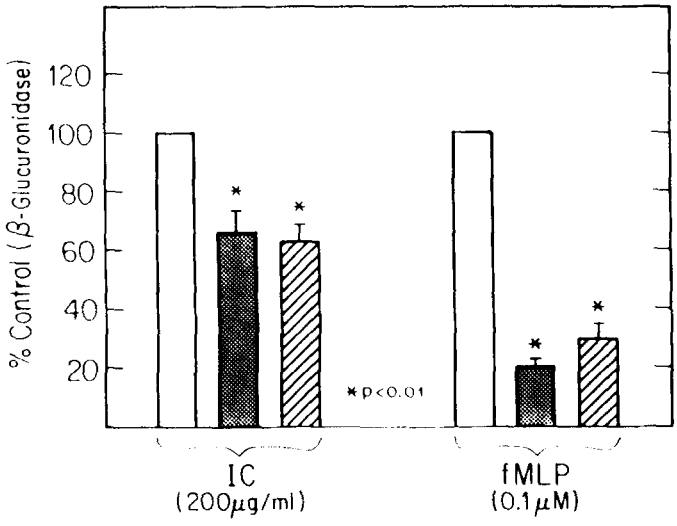

Figure 4. Effect of pertussis toxin and colchicine on degranulation. *Open bars* represent control response in the absence of pertussis toxin. Cells were preincubated with pertussis toxin (400 ng/ml, 90 min) and exposed to buffer (□), or colchicine (▨) (45 min). Cells were stimulated with IC or FMLP. β-Glucuronidase was measured as described previously and results are expressed as percent of control response. In this set of experiments, cells released a mean of 6.5% ± 1.0 of total β-glucuronidase in response to IC and 21.2 ± 1.2% in response to FMLP after subtraction of background (3.0 ± 0.9%).

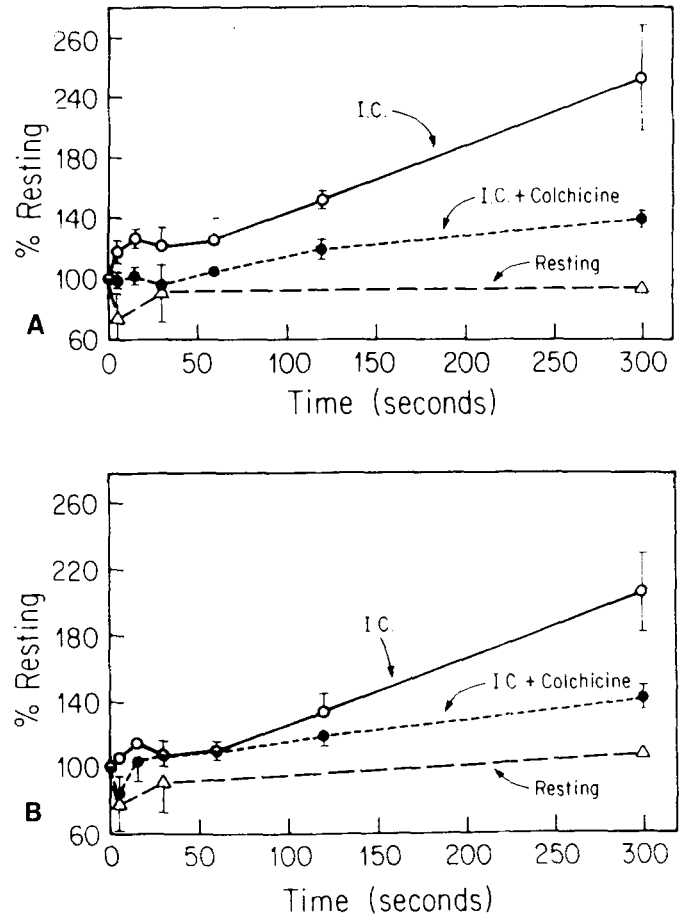

Figure 5. Effect of colchicine on the formation of [^{3}H]AA-DG and diacyl[^{14}C]glycerol after IC. Cells were prelabeled with [^{3}H]arachidonate and [^{14}C]glycerol as described in *Methods*. Cells were then incubated in the absence (–) or presence (– – – –) of colchicine, stimulated with IC, and 1,2-*sn*-DG was isolated by TLC as described in *Methods*. Results are expressed as percent of resting levels for [^{3}H]AA-DG (*a*) and diacyl[^{14}C] glycerol (*b*).

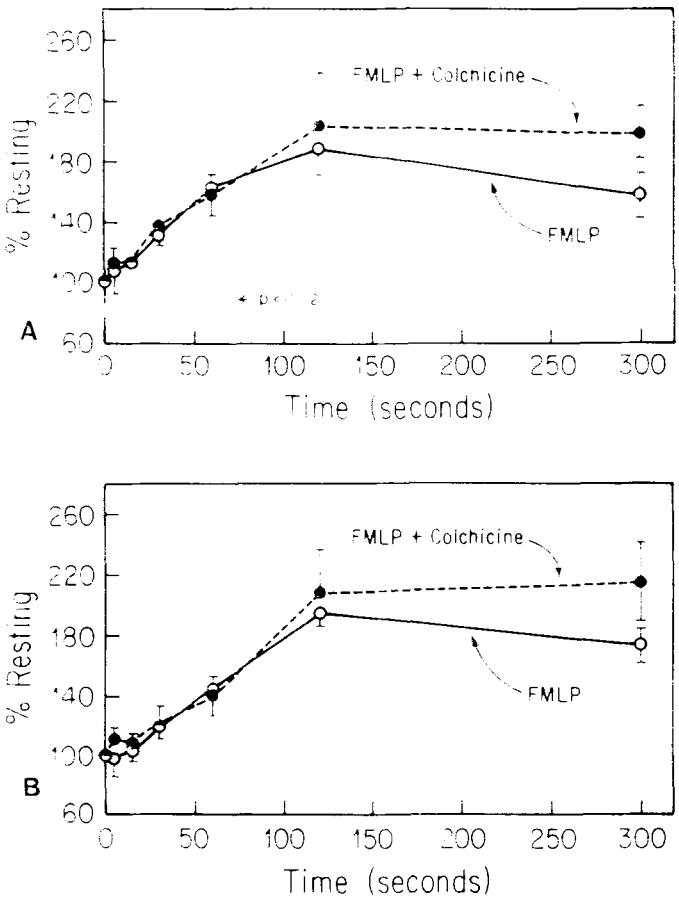

Figure 6. Effect of colchicine on the formation of [^{3}H]AA-DG and diacyl[^{14}C]glycerol after FMLP. Cells were prelabeled with [^{3}H]arachidonate and [^{14}C]glycerol as described in *Methods*. Cells were then incubated in the absence (–) or presence (– – – –) of colchicine, stimulated with FMLP, and 1,2-*sn*-DG was isolated by TLC as described in *Methods*. Results are expressed as percent of resting levels for [^{3}H]AA-DG (*a*) and diacyl[^{14}C]glycerol (*b*).

DISCUSSION

Our studies demonstrate that intact MT are not required for neutrophils to degranulate. Whereas optimal degranulation elicited by engagement of Fcγ receptors requires intact MT, that elicited by FMLP does not. Thus the role played by MT in degranulation of neutrophils is dependent on the stimulus used for activation.

Previous studies suggest that during cell activation, granules of neutrophils move via an energy-driven mechanism along pathways directed by polymerized MT. Upon exposure of neutrophils to a variety of stimuli (zymosan-treated serum, Con A, FMLP, Ca^{2+} ionophore) MT increase in both length and number (19, 22, 23, 26, 29, 55, 56). Furthermore, the inhibition of MT assembly has been reported to decrease degranulation elicited by heat-killed staphylococci, zymosan-treated serum, and IC (22, 23, 28, 29, 57). Moreover, microscopic examination of neutrophils reveals a close association between granules and MT that is mediated by cross-bridges. These cross-bridges may represent the ATPase and the kinesin that have been identified in association with both MT and granules (17, 22).

The data presented here give evidence to the contrary; whereas intact MT were required for optimal degranulation elicited by IC, this was not so for degranulation elicited by FMLP. Thus the translocation of granules or plasmalemma leading to phagolysosome fusion during the immediate degranulation response cannot require intact MT. Indeed, whereas Rothwell et al. (17) demonstrate that FMLP causes an increase in the number of granules associated with MT, they do not demonstrate that FMLP causes a difference in the direction of the movement of granules along MT. The role of the close apposition of granules with MT visualized by electron microscopy remains an enigma. We now hypothesize that MT, rather than acting uniformly as "motors" for granule movement during exocytosis, act on the signal transduction required for this process. MT may participate in the organization and cycling of specific receptors, the activation of the pertussis toxin-sensitive phospholipase C, or the generation of 1,2-*sn*-DG.

MT may alter the mobility of specific receptors in the membrane. Indeed, MT assembly determines the distribution of lectin-binding sites in lymphocytes and neutrophils (58, 59). In contrast, modulation of the FMLP receptor is not associated with MT (60, 61). Thus the failure of agents that depolymerize MT to inhibit degranulation elicited by FMLP may be explained on this basis. Whereas little is known about the relation of Fcγ receptors to MT, it could be postulated that the cycling of specific Fcγ receptors is modulated by the state of MT assembly.

Neutrophils contain primarily two Fcγ receptors: FcγRII (40 kDa, 10,000 to 20,000 sites/cell) and FcγRIII (50 to 80 kDa, 100,000 to 200,000 sites/cell) (39, 40, 62). It has not yet been elucidated whether these molecules have overlapping functions. Whereas ligation of FcγRII elicits O_2^- generation in neutrophils by a pertussis toxin-sensitive pathway, ligation of FcγRII molecules elicits degranulation by both a pertussis toxin-sensitive and -insensitive pathway (53). FcγRIII molecules of neutrophils bind IC thus mediating rosetting of E (39). The ligation of these molecules also triggers lysis of E (63) but not hybridoma cells that have been coated with anti-FcγRIII-F(ab) conjugated to anti-chicken F(ab) (64). It remains to be determined whether FcγRIII also elicits signals for cell activation (degranulation and O_2^- generation).

We now demonstrate that blocking FcγRIII sites with mAb 3G8 significantly inhibits the release of both β-glucuronidase and lysozyme. Thus FcγRIII participates in degranulation. However, it is not clear whether FcγRIII participates in degranulation by enhancing the binding of IC or whether ligation of FcRIII triggers the generation of intracellular signals. Moreover, despite removal of a large proportion of FcγRIII molecules by PI-PLC, signaling, as measured by the release of lysosomal enzymes, proceeds almost normally. Human FcγRIII has a phosphatidylinositol anchor in neutrophils and is polymorphic with two alloantigens NA1 and NA2 (43). These two allotypes are encoded by cDNA that differ by five nucleotides, which predict four amino acid substitutions and cause differences in N-linked glycosylation sites (45, 46, 65). It is unknown whether these differences account for differences in the sensitivity of FcγRIII molecules to PI-PLC. Since treatment of cells with PI-PLC did not inhibit degranulation, we hypothesize that molecules of FcγRIII that are released by PI-PLC may be able to elicit intracellular signals that differ from that of molecules that remain. An alternative hypothesis is that engagement of only a small percentage of FcγRIII molecules may be required for degranulation.

MT may participate in the generation of specific intracellular signals. Activation of neutrophils proceeds after binding of GTP to one or several pertussis toxin-sensitive GTP-binding proteins resulting in phospholipid remodeling and the activation of protein kinases (50, 66–70). Ligation of FcγRII activates neutrophils by both a pertussis toxin-sensitive and -insensitive pathway (53). The data presented here suggest that MT alter the generation of signals via an effect on the pertussis toxin-sensitive pathway since depolymerization of MT did not further the inhibition of degranulation elicited by pertussis toxin. However, O_2^- generation, which proceeds after activation of a pertussis toxin-sensitive G_{pc}, was not altered by depolymerization of MT. Recent evidence suggests that there are multiple GBP in neutrophils, cytosolic as well as membrane-bound (71–75). The mechanism by which these GBP associate with other components of the activation pathway is not known. Topical localization of specific GBP may provide a mechanism for the control over cell activation. It is clear from our data that the activation of the GBP in the cytosol involved in the assembly of the respiratory burst oxidase is not dependent on MT integrity. In contrast, the guanine nucleotide-binding proteins associated with granules may be influenced by the polymerized state of MT.

MT may participate in phospholipid remodeling by affecting signals generated by engagement of receptors, by maintaining lipid domains in the plasma membrane, or by directly altering lipid synthesis in cells (76). We have previously demonstrated that disassembly of MT inhibits the formation of 5-lipoxygenase products (19) most likely by inhibiting the translocation of the 5-lipoxygenase (77, 78). We now demonstrate that the generation of 1,2-*sn*-DG is altered by the state of MT polymerization in neutrophils. We have previously demonstrated a biphasic increase in 1,2-*sn*-DG during neutrophil activation elicited by FMLP in the presence of cytochalasin B; an "early" increase derived from the hydrolysis of inositol phosphates (triggering) and a "late" increase (activation) derived from some other source (4–6). With the use of the novel probe, protein I, a porin derived from *Neisseria gonorrhoeae*, we have demonstrated an association of the "late" (activation) rise in 1,2-*sn*-DG with degranulation (5). In the current studies, the "late" rise in 1,2-*sn*-DG elicited by IC was more gradual than that demonstrated after treatment of cells with FMLP, a result that correlates with the effect of these stimuli on degranulation; whereas IC elicited release of 10.3 ± 3% of total β-glucuronidase at 5 min, FMLP elicited release of 26.3 ± 1.5%. In contrast, the increase in 1,2-*sn*-DG elicited by these two stimuli did not correspond to their ability to generate O_2^-; treatment of neutrophils with IC or FMLP elicited the reduction of 21.5 ± 2.5 or 29.2 ± 2.7 nm of cytochrome *c*, respectively. The "late" generation of both [^{3}H]AA-DG and diacyl[^{14}C]glycerol formed in response to IC was decreased by depolymerization of MT with colchicine. In contrast, [^{3}H]AA-DG and diacyl[^{14}C]glycerol formed in response to FMLP was increased at the "late" time point. These changes parallel the effect of colchicine on degranulation. Since colchicine had no effect on the generation of O_2^-, these studies underscore the disassociation between the "late" (activation) rise in 1,2-*sn*-DG with the generation of O_2^-. The "late" (activation) increase may be secondary to the generation of 1-*O*-alkyl diglyceride, which is involved in "priming" of neutrophils (79, 80). The alterations in 1,2-*sn*-DG demonstrated in our studies with colchicine are primarily "late," thus although we have not yet directly measured the generation of 1-*O*-alkyl-2-acylglycerols, it is possible that MT also alter the generation of these moieties.

Alternatively, MT may be required for a specific mode of intracellular transport of proteins. Indeed in both the mouse pituitary tumor cell line (At20) and endothelial cells, the targeting of proteins utilized in regulated secretory pathways is MT-dependent, whereas the release of constitutively secreted proteins is not (81–84). Furthermore, in neutrophils as in other secretory cells, MT may be required only for polarized secretion of proteins; polarized Madin-Darby canine kidney cells require intact MT for insertion of hemagglutinin glycoprotein to their apical surface but not for the insertion of this protein to their basolateral surface (85). Our system consists of cells in suspension. These cells are not polarized when exposed to soluble stimuli such as FMLP, but do polarize and spread along the insoluble IC. Neutrophils may require intact MT for this polarized release of lysosomal enzymes, thus explaining the selective effect of MT disassembly on secretion provoked by IC.

The data demonstrate that degranulation elicited by IC, but not FMLP, is optimal in the presence of intact MT. Thus MT cannot universally be required for the association of granules with the plasmalemma during secretion as previously hypothesized. Moreover, the assembly of the respiratory burst oxidase and thus O_2^- generation proceeds regardless of the state of MT assembly. In contrast, optimal signaling for degranulation elicited by engagement of FcγRII and FcγRIII requires intact MT. MT most likely alter the cycling of specific receptors and thus their ability to initiate phospholipid remodeling, kinase activation, and subsequent degranulation. The requirements for degranulation and O_2^- generation differ in these respects.

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