Fc Receptor Stimulation of Phosphatidylinositol 3-Kinase in Natural Killer Cells Is Associated with Protein Kinase C–independent Granule Release and Cell-mediated Cytotoxicity

By Joy D. Bonnema, Larry M. Karnitz, Renee A. Schoon, Robert T. Abraham, and Paul J. Leibson

From the Department of Immunology, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Summary

Although diverse signaling events are initiated by stimulation of multichain immune recognition receptors on lymphocytes, it remains unclear as to which specific signal transduction pathways are functionally linked to granule exocytosis and cellular cytotoxicity. In the case of natural killer (NK) cells, it has been presumed that the rapid activation of protein kinase C (PKC) enables them to mediate antibody-dependent cellular cytotoxicity (ADCC) and "natural" cytotoxicity toward tumor cells. However, using cloned human NK cells, we determined here that Fc receptor stimulation triggers granule release and ADCC through a PKC-independent pathway. Specifically, pretreatment of NK cells with the selective PKC inhibitor, GF109203X (using concentrations that fully blocked phorbol myristate acetate/ionomycin–induced secretion) had no effect on FcR-initiated granule release or ADCC. In contrast, FcR ligation led to the rapid activation of phosphatidylinositol 3-kinase (PI 3-kinase), and inhibition of this enzyme with the selective inhibitor, wortmannin, blocked FcR-induced granule release and ADCC. Additional experiments showed that, whereas FcR-initiated killing was wortmannin sensitive and GF109203X insensitive, natural cytotoxic activity toward the tumor cell line K562 was wortmannin insensitive and GF109203X sensitive. Taken together, these results suggest that: (*a*) PI 3-kinase activation induced by FcR ligation is functionally coupled to granule exocytosis and ADCC; and (*b*) the signaling pathways involved in ADCC vs natural cytotoxicity are distinct.

A critical event in the development of cell-mediated cytotoxicity is the receptor-stimulated release of granule-derived proteins (1). Before cytotoxic lymphocyte activation, preformed granules containing an array of cytotoxic and proteolytic molecules reside beneath the cell surface. Upon receptor recognition of susceptible targets, intracellular signals direct the transport of the granules toward the effector cell–target cell interface where they fuse with the plasma membrane and release their mediators. Previous work has focused on the role of phospholipase C (PLC)[1]–derived signals in regulating NK cell and CTL secretory responses (for reviews, see references 2–5).

Specifically, protein tyrosine kinase (PTK)-dependent activation of PLC is initiated after stimulation of NK cells through their FcR (6–9) or of T lymphocytes through their TCR complex (10–14). The subsequent PLC-catalyzed hydrolysis of phosphoinositides generates *sn*-1,2-diacylglycerol and inositol-1,4,5-trisphosphate, which in turn mediate the activation of PKC and the mobilization of intracellular calcium, respectively. Because PKC-activating phorbol esters, acting in conjunction with calcium ionophores, can induce granule release from NK cells and CTL (15–17), it has been presumed that secretion initiated by cross-linking of multichain immune recognition receptors on these cell types is mediated via PKC-dependent pathways. Furthermore, it has been presumed that the proximal signals controlling FcR- or TCR-initiated secretion would be similar to those inducing granule release after NK cell recognition of susceptible tumor targets. Direct evidence for either of these assumptions is lacking.

Recently, it has been shown that FcγRIII- or TCR-initiated activation of PTK also results in the induction of a phosphatidylinositol 3-kinase (PI 3-kinase)–dependent pathway (18–20). PI 3-kinase is a heterodimer composed of a catalytic p110 subunit and a regulatory p85 subunit (21–26). Upon activation, PI 3-kinase phosphorylates phosphoinositides at the D-3 position of the inositol ring to generate such prod-

[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; PI 3-kinase, phosphatidylinositol 3-kinase; PIP, phosphatidylinositol-4-phosphate; PKC, protein kinase C; PLC, phospholipase C; and PTK, protein tyrosine kinase.

ucts as phosphatidylinositol 3-phosphate, phosphatidylinositol 3,4-bisphosphate, and phosphatidylinositol 3,4,5-trisphosphate (27). These phosphoinositides are distinct from those used in the classical PLC-dependent signaling pathway, and it is therefore presumed that the lipids themselves are important molecules in signal transduction pathways. Specific attention has recently focused on the effects of PI 3-kinase on the sorting of vacuoles in yeast (28), platelet-derived growth factor (PDGF) receptor endocytosis (29), histamine release from mast cells (30), and cytoskeleton changes in thrombin-stimulated platelets (31). Taken together, these experimental observations suggest a potential general role for PI 3-kinase in the regulation of vesicular transport.

To investigate whether PKC- and/or PI 3-kinase–dependent pathways regulate granule release from cytotoxic lymphocytes, one would like to selectively interrupt each pathway and determine the subsequent effects on receptor-initiated secretion. With the recent identification of the bisindolylmaleimide GF109203X (32) and the microbial metabolite wortmannin (30) as selective PKC and PI 3-kinase inhibitors, respectively, this experimental strategy is now possible. Using cloned human NK cells, we report here that FcγRIII-initiated granule release and antibody-dependent cellular cytotoxicity (ADCC) are, in fact, regulated by PKC-independent pathways. Rather, the profound inhibition of these functions by wortmannin suggests that FcR-initiated activation of PI 3-kinase modulates granule release from these cells. In contrast, the inability of wortmannin to inhibit direct antitumor NK cell–mediated cytotoxicity suggests that alternative signaling pathways are used during this mode of killing.

Materials and Methods

Chemical Reagents and Antibodies. Ionomycin and GF109203X were obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Streptolysin-O was purchased from Wellcome Diagnostics (Dartford, England). All other chemicals, unless otherwise noted, were obtained from Sigma Chemical Co. (St. Louis, MO). The anti-FcRγIII mAb 3G8 (anti-CD16) (33) were purified by affinity chromatography over protein A agarose. $F(ab')_2$ fragments of goat anti-mouse IgG were purchased from Organon Teknika (West Chester, PA). The antiphosphotyrosine antibody-secreting hybridoma, 1G2, was a gift from A. R. Frackelton, Jr. (Brown University, Providence, RI). 1G2 mAb purification has been previously described (34). The rabbit anti-p85 antisera was raised against a GST-p85 fusion protein containing p85 amino acid residues 158-724.

Cell Lines. K562 (human erythroleukemia cell line) and P815 (murine mastocytoma cell line) were obtained from American Type Culture Collection (Rockville, MD). The anti-CD16 3G8 hybridoma was generously provided by D. M. Segal (National Cancer Institute/National Institutes of Health, Bethesda, MD). These cell lines were maintained in RPMI 1640 supplemented with 10% bovine calf serum. Human PBL were isolated from the defibrinated blood of healthy donors by Ficoll-Hypaque density gradient centrifugation. Human $CD16^+$ NK cell lines were isolated and characterized as previously described (6).

Cell Permeabilization. NK cell permeabilization was performed as previously described (17). Briefly, NK cells were washed twice in Ca^{2+} and Mg^{2+}-free HBSS and resuspended in permeabilization buffer (40 mM Pipes, 1.5 mM EGTA, 5 mM Na_2HPO_4, 10 mM $MgCl_2$, 80 mM potassium glutamate, 5 mM NaCl, 0.1 mM dithiothreitol, 0.5 mg/ml BSA, and 3 mM ATP, pH 7.2). The amount of $CaCl_2$ required to yield the indicated concentration of free Ca^{2+} in the permeabilized buffer was calculated with the free calcium algorithm. For each sample (total reaction volume, 250 μl), 7.5 × 10^5 NK cells were mixed with the indicated stimuli and streptolysin O (0.4 U/ml). The reaction mixtures were incubated at 37°C for 30 min and then centrifuged at 500 *g* for 10 min. 200 μl of the supernatant was collected from each sample for subsequent quantitation by the secretion assay.

Secretion Assay. As previously described (17), a fluorescence assay was used to quantitate exocytotic release of the granule-derived enzyme, hexosaminidase, from NK cells. Briefly, duplicate sample supernatants (50 μl each) were diluted 1:2 in 250 mM sucrose, 3 mM imidazole, 0.1% ethanol, pH 7.4, and then incubated for 20 min at 37°C with 100 μl of 0.5 mM 4-methylumbelliferyl-2-acetamide-2-deoxy-β-D-glucopyranoside (Research Products International Corp., Prospect, IL) in 0.1 M sodium citrate and 0.2% Triton X-100. Reactions were terminated by the addition of 2 ml 50 mM glycine–5 mM EDTA, pH 10.4. Fluorescence was measured with a digital fluorometer (model 450; Sequoia-Turner, Mountain View, CA) at excitation and emission wavelengths of 365 and 450 nm, respectively. For each experiment, total intracellular hexosaminidase content was measured after cell lysis with 1% Triton X-100. Percent secretion was calculated as: 100 × [(stimulated release − spontaneous release)/(maximum release − spontaneous release)]. Spontaneous release for all experiments was <20% of maximum release. The range of duplicate samples was <10% of the mean.

Cytotoxicity Assay. The ^{51}Cr-release assays measuring either direct NK cell–mediated cytotoxicity or ADCC were performed as previously described (6). Spontaneous release was <15% of the maximum release for all experiments. For results expressed as lytic units, 1 lytic unit is the number of cells required to give 20% specific ^{51}Cr release.

PI 3-Kinase Assay. NK cell clones were cultured in RPMI 1640 medium supplemented with 10% bovine calf serum for 16 h before analysis. For each sample, 10^7 cells were treated as noted in figure legends, then diluted in ice cold lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 40 mM β-glycerophosphate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml pepstatin A, and 1 mM PMSF). The cleared lysates were immunoprecipitated with either anti-p85 antibody bound to protein A–Sepharose beads, or 1G2 antibody covalently bound to Sepharose beads. After 45 min at 4°C, the immunoprecipitates were washed three times with wash buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 40 mM β-glycerophosphate, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, and 200 μM adenosine) and twice with 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM Na_3VO_4, and 200 μM adenosine. PI-3 kinase assays were performed as previously described (35). The phosphatidylinositol-4-phosphate (PIP) standard was chromatographed in parallel sample lanes and visualized by staining with iodine vapors. Radiolabeled phosphoinositides were detected by autoradiography. In addition, ^{32}P was quantitated after scanning the TLC plates using the Radioanalytic Imaging System (model 4000, AMBIS, Inc., San Diego, CA).

Results

PKC-independent Regulation of FcR-induced Secretion. It remains unclear which signals generated after FcR ligation

regulate granule exocytosis from NK cells. Because direct pharmacologic activation of PKC can induce secretion from NK cells (16, 17), we first evaluated whether PKC plays a similar role after FcR stimulation. We have previously shown that stimulation of NK cells with a variety of secretagogues induces the coordinate release of hexosaminidase and *N*-α-benzyloxycarboxy-L-lysine thiobenzyl ester (BLT)-esterase (17). Because the fluorescence assay for hexosaminidase has proven to be the more sensitive method for measuring low levels of granule exocytosis from human NK cells (17), results from the hexosaminidase assays are reported in this study. Cloned $CD16^+$, $CD3^-$ NK cells were first exposed to varying concentrations of the selective PKC inhibitor GF109203X (32), and then stimulated with either 3G8 mAb (anti-FcγRIII)-coated polystyrene beads or a combination of the PKC-activating phorbol ester, PMA, and the calcium ionophore, ionomycin. As shown in the top panel of Fig. 1, pretreatment of NK cells with concentrations of GF109203X that fully inhibited PMA/ionomycin–induced secretion did not alter FcR-induced granule release. These results suggest that although PKC activation can result in granule exocytosis from NK cells, FcR-stimulated granule release is regulated by PKC-independent signaling pathways.

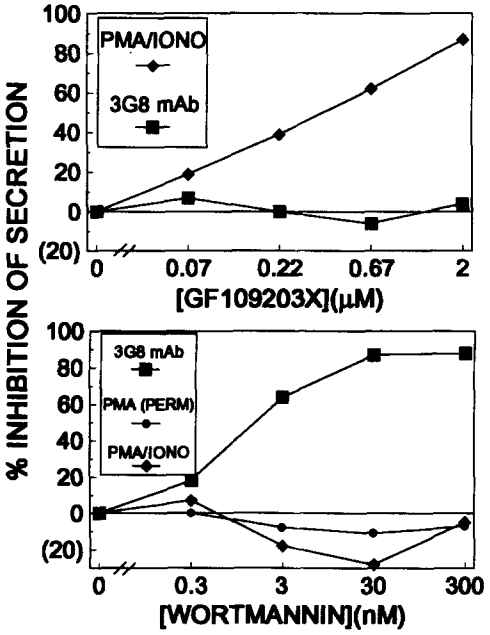

Figure 1. Selective inhibition of FcR-induced secretion by wortmannin. Cloned $CD16^+$, $CD3^-$ human NK cells (7.5×10^5 cells/sample) were preincubated for 15 min at 37°C with the indicated concentrations of either GF109203X (*top*) or wortmannin (*bottom*). (*Top*) GF109203X-treated cells were then incubated for an additional 3 h with either anti-FcγRIII mAb (*3G8*)-coated, 6-μM-diameter polystyrene beads (■) or PMA (1 ng/ml) and ionomycin (1 μM) (◆). Hexosaminidase activity released into the supernatants was quantitated by measuring the conversion of 4-methylumbelliferyl-2-acetamide-2-deoxy-β-D-glucopyranoside into the fluorescent 4-methylumbelliferone product. Without GF109203X pretreatment, the NK cells stimulated with anti-FcγRIII-coated beads or PMA/ionomycin released 6.4 and 20.2% of their total intracellular hexosaminidase content, respectively. (*Bottom*) Wortmannin-treated cells were stimulated with either anti-FcγRIII mAb (*3G8*)-coated beads (■), PMA (2 ng/ml), and ionomycin (1 μM) (◆), or PMA (1 ng/ml) and streptolysin-0 (0.4 U/ml) (●). For streptolysin O-permeabilized cells, free calcium concentration was maintained at 10 μM. Intact cells and streptolysin O–permeabilized cells were incubated at 37°C with the agonists for 3 and 1 h, respectively. Without wortmannin pretreatment, the NK cells stimulated with anti-FcγRIII–coated beads, PMA/ionomycin, or PMA/streptolysin-O released 6.4, 11.4, and 38.4% of their total intracellular hexosaminidase content, respectively.

FcR-dependent Granule Release from NK Cells Is Blocked by the PI 3-Kinase Inhibitor, Wortmannin. The novel finding that FcR-induced granule exocytosis is not regulated by PKC prompted us to investigate whether PI 3-kinase, a potential modulator of vesicular transport (28–31), might influence secretion from NK cells. Because the p85 regulatory subunit of PI 3-kinase can be tyrosine phosphorylated during cellular activation and also has an SH2 domain that binds to tyrosine phosphorylated proteins during cellular activation (27, 36–39), antiphosphotyrosine immunoprecipitates from FcR-stimulated NK cells were assayed for PI 3-kinase activity. Consistent with the recent report by Kanakaraj et al. (18), we found that FcγRIII stimulation of NK cells increased phosphotyrosine-associated PI 3-kinase activity (Fig. 2). Specifically, PI 3-kinase activity increased within 2 min of FcR ligation, peaked at 10 mins (sixfold increase), and declined by 30 min.

To evaluate the functional consequences of this PI 3-kinase activation, we characterized the effects of a recently described PI 3-kinase inhibitor, wortmannin (30), in this experimental system. Wortmannin, a fungal metabolite, has been shown to specifically and irreversibly inhibit PI 3-kinase activity at nanomolar concentrations by binding to the p110 catalytic subunit of PI 3-kinase (30). To evaluate its effects on cloned human NK cells, lysates from drug-treated cells were immunoprecipitated with anti-p85 antibodies and the immunoprecipitates were assayed for PI 3-kinase catalytic activity (Fig. 3). Consistent with concentration–inhibition relationships observed in other cell types (30), wortmannin inhibited PI 3-kinase with an IC_{50} of ~3 nM. Concentrations of wortmannin that fully inhibited PI 3-kinase activity in these NK cells had no effect on conjugate formation with susceptible targets (as quantitated by flow cytometry) or on FcR-initiated tyrosine kinase activation (as detected by antiphosphotyrosine immunoblotting) (data not shown). In parallel studies, pretreatment of NK cells with wortmannin potently inhibited FcR-induced secretion in a concentration-dependent manner with an IC_{50} of ~2 nM (Fig. 1, *bottom*). It is important to note that although wortmannin blocked FcR-induced granule release from NK cells, PKC-dependent release from either intact cells stimulated with PMA/ionomycin or permeabilized cells stimulated with PMA in buffer containing high free Ca^{2+} concentration was not inhibited by wortmannin (Fig. 1, *bottom*). These data, together with the differential effects of GF109203X on NK cell secretion (Fig. 1, *top*) suggest that FcR-induced secretion is regulated by a PI 3-kinase–dependent, PKC-independent pathway. Conversely, pharmacologic activation by phorbol esters induces secretion via a separate PKC-dependent, PI 3-kinase–independent pathway.

FcR-dependent Cell-mediated Cytotoxicity Is Blocked by the

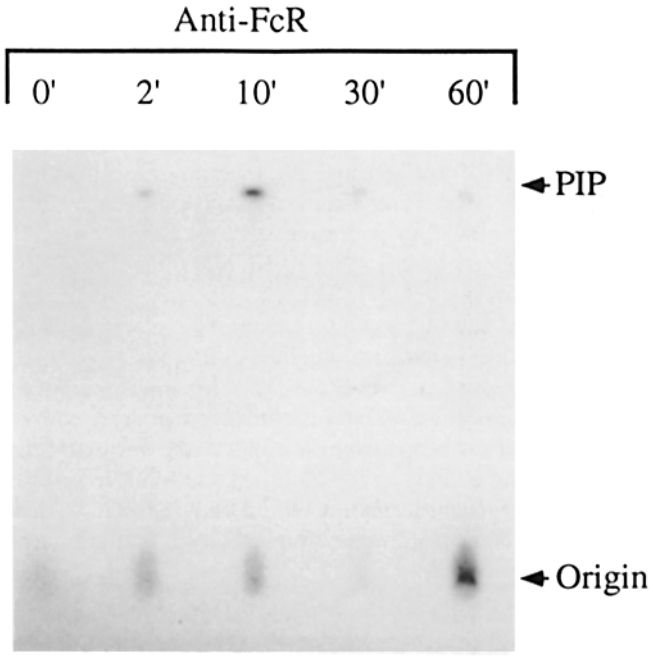

Figure 2. FcR stimulation induces activation of PI 3-kinase in NK cells. Aliquots (10^7 cells/sample) of cloned NK cells that had been incubated without IL-2 for 16 h were stimulated at 37°C for the time indicated with anti-FcγRIII mAb (3G8; 10 μg/ml) cross-linked with goat $F(ab')_2$ fragments of anti-mouse IgG. Antiphosphotyrosine (IG2 mAb) immunoprecipitates were assayed for PI 3-kinase activity, using phosphatidylinositol as substrate. Phosphoinositides were separated by TLC and radiolabeled products were visualized by autoradiography. (*Top arrow*) Position of a PIP standard that was chromatographed in a parallel lane.

PI 3-kinase Inhibitor, Wortmannin. Because FcR-induced granule exocytosis is believed to deliver the "lethal hit" during ADCC, we performed parallel analyses evaluating the potential roles of PI 3-kinase and PKC in regulating FcγRIII-dependent, cell-mediated killing. Pretreatment of cloned NK cells with wortmannin inhibited killing of both the anti-FcγRIII mAb–secreting hybridoma, 3G8 (Fig. 4, *top*), and the P815 mastocytoma cell line that is normally rendered sensitive to NK cell–mediated cytotoxicity by coating with 3G8 mAb ("reverse ADCC") (Fig. 4, *bottom*). Similar to the IC_{50} for wortmannin's inhibition of FcR-induced granule release, wortmannin inhibited FcR-dependent killing of each target cell

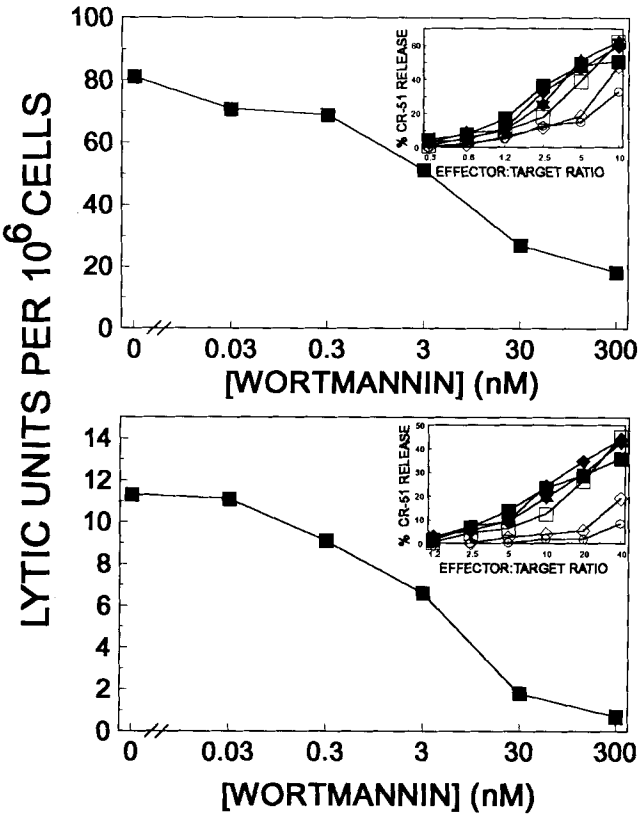

Figure 4. Wortmannin inhibits FcR-dependent NK cell–mediated cytotoxicity. Cloned NK cells were preincubated for 15 min at 37°C with the indicated concentrations of wortmannin, washed, and then incubated for 1 h at 37°C with either ^{51}Cr-labeled 3G8 (anti-FcγRIII) hybridoma cells (*top*) or ^{51}Cr-labeled P815 cells bound to 3G8 mAb (*bottom*). Results are expressed as specific ^{51}Cr release in the insets ([■] no wortmannin, [◆] 0.03 nM wortmannin, [★] 0.3 nM wortmannin, [□], 3 nM wortmannin, [◇] 30 nM wortmannin, and [○] 300 nM wortmannin) or as lytic units per 10^6 cells, where 1 lytic unit is the number of cells required to give 20% specific ^{51}Cr release.

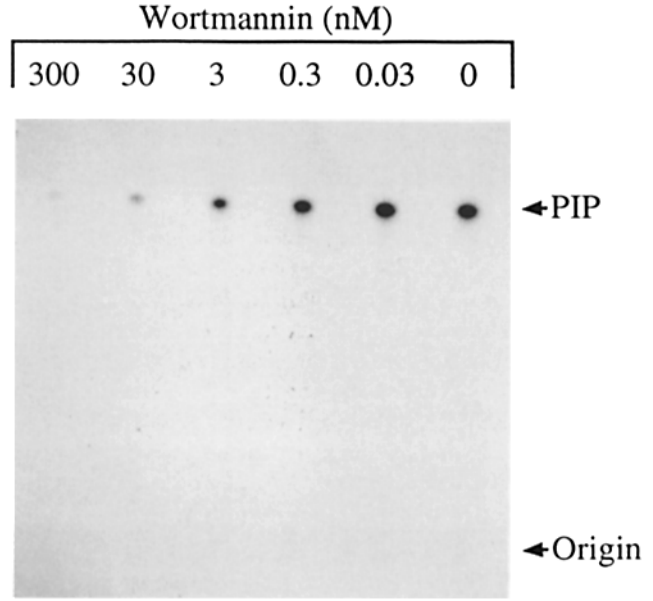

Figure 3. Inhibition of PI 3-kinase in wortmannin-treated NK cells. Cloned NK cells (10^7 cells/sample) were incubated for 30 min at 37°C with the indicated concentrations of wortmannin. Anti-p85 immunoprecipitates from cell lysates were then assayed for PI 3-kinase activity. Radiolabeled phosphoinositides were separated by TLC, visualized by autoradiography, and quantitated using an Ambis 4000 Radioanalytic Imaging System. Radiolabel in phosphatidylinositol phosphate products from NK cells treated with 300, 30, 3, 0.3, 0.03, or 0 nM wortmannin was 39, 190, 535, 1,005, 1,443, or 1,250 cpm, respectively.

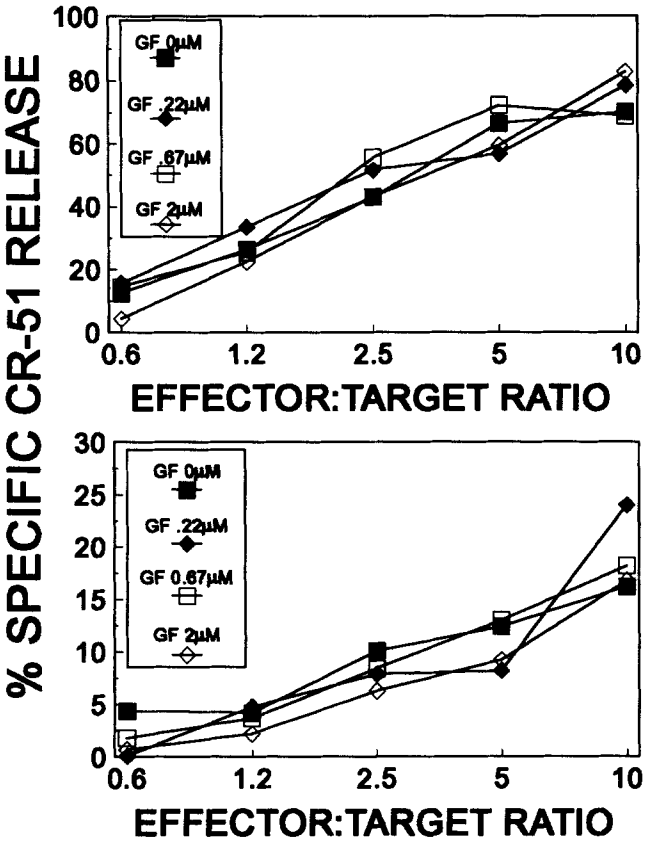

Figure 5. The PKC inhibitor, GF109203X, fails to inhibit FcR-dependent NK cell–mediated cytotoxicity. Cloned NK cells were pretreated for 15 min at 37°C with the indicated concentrations of GF109203X and the incubated for 1 h at 37°C with either ^{51}Cr-labeled 3G8 (anti-FcγRIII) hybridoma cells (*top*) or ^{51}Cr-labeled P815 cells bound to 3G8 mAb (*bottom*).

type (i.e., 3G8 hybridomas or 3G8 mAb–coated P815 cells) with IC_{50}'s of ~3 nM. Wortmannin also inhibited FcR-dependent killing mediated by either clonal NK cell lines, polyclonal NK cell lines, or NK cells from PBL (data not shown). In repeated experiments, the percent inhibition by wortmannin was consistently less for freshly isolated NK cells than for IL-2–dependent NK cell lines. In contrast to the ability of the PI 3-kinase inhibitor, wortmannin, to reduce FcR-dependent NK cell–mediated killing, the PKC inhibitor, GF109203X, did not affect this mode of cellular cytotoxicity, as measured against either ^{51}Cr-labeled 3G8 hybridoma cells (Fig. 5, *top*) or 3G8 mAb-coated P815 cells (Fig. 5, *bottom*). Taken together, these data suggest that FcR stimulation of NK cell granule release and cell-mediated cytotoxicity is selectively blocked by the PI 3-kinase inhibitor, wortmannin.

Separate Signaling Pathways Used during Alternative Modes of NK Cell–mediated Cytotoxicity. NK cells can lyse target cells either by FcR-dependent cytotoxicity of IgG-coated target cells (i.e., ADCC) or by direct antitumor cell-mediated killing. In both cases, granule release from the NK cells appears to be required for effective killing. However, it remains unclear whether the proximal signals functionally linked to granule release are the same or distinct. Having demonstrated that FcR-dependent cytotoxicity was blocked by the PI 3-kinase inhibitor, wortmannin, but not by the PKC inhibitor, GF109203X, we extended our analysis with these pharmacologic inhibitors to direct NK cell–mediated killing of the prototypical NK-sensitive tumor target, K562. Whereas FcR-dependent killing of the hybridoma, 3G8, was wortmannin sensitive (Fig. 6 *A*, *top*), and GF109203X resistant (Fig. 6 *B*, *top*), killing of K562 was wortmannin resistant (Fig. 6 *A*, *bottom*) and GF109203X sensitive (Fig. 6 *B*, *bottom*). These data suggest that although PI 3-kinase activation appears to

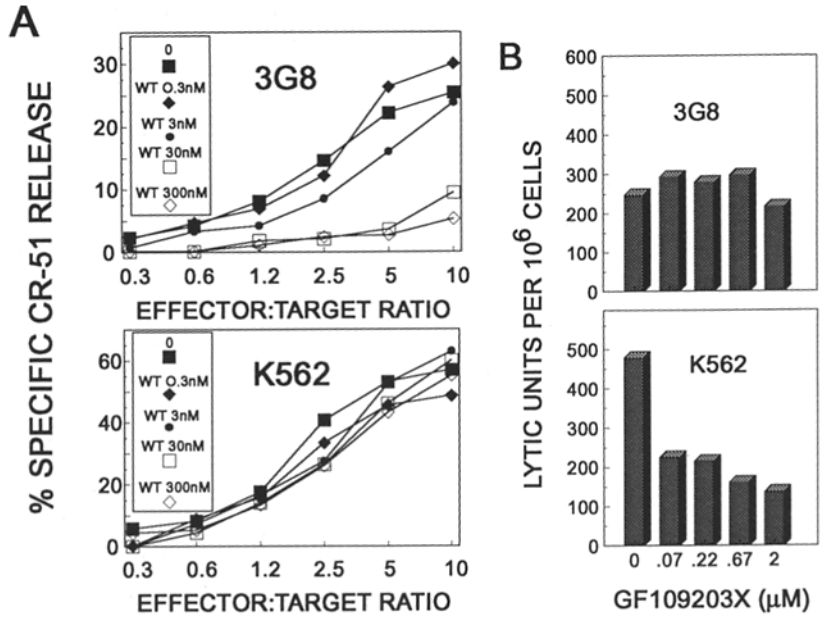

Figure 6. FcR-dependent cytotoxicity and direct antitumor cytotoxicity are regulated by separate signaling pathways. Cloned NK cells were preincubated for 15 min at 37°C with the indicated concentrations of either wortmannin (*A*) or GF109203X (*B*). Drug-treated cells were then incubated for 1 h with either ^{51}Cr-labeled 3G8 (anti-FcγRIII) hybridoma cells (*top*) or ^{51}Cr-labeled K562 tumor cells (*bottom*).

be critical for FcR-induced granule release and ADCC, a separate PKC-dependent pathway appears to regulate direct NK cell–mediated antitumor killing.

Discussion

Cytotoxic lymphocyte recognition of susceptible target cells elicits a diverse array of second messengers and the subsequent development of pleiotropic responses, including effects on granule release, cellular cytotoxicity, cytokine gene transcription, and cell surface receptor expression (2–5, 40, 41). The heterogeneity of these biological responses has complicated attempts to link distinct proximal signaling events with specific downstream functional responses. In this study, we selectively inhibited separate signaling pathways generated after FcR ligation in NK cells and characterized the resulting effect on granule release and FcR-dependent cytotoxicity. Our results suggest that FcR stimulation of PI 3-kinase in NK cells is associated with both granule exocytosis and the generation of cellular cytotoxicity.

Early studies focused on the PTK-catalyzed activation of PLC as a central event during lymphocyte activation (for reviews, see references 2–5). With the subsequent demonstration that PKC-activating phorbol esters together with calcium ionophores could induce granule release from NK and T cells (15–17), the consensus view was that FcR- and TCR-initiated secretory responses are controlled by PKC-dependent pathways. This notion was further bolstered by demonstrations that PKC could regulate granule exocytosis in other secretory cell types (e.g., mast cells, chromaffin cells, etc.) and could phosphorylate granule-associated proteins (42–45). To determine whether FcR- or TCR-initiated granule release is, in fact, mediated via a PKC-dependent pathway, one would like to assess the effects of selective and potent PKC inhibitors on this process. However, to date, most of the reported PKC inhibitors display poor selectivity both in vitro and in vivo. For example, H7 inhibits with similar potency PKC, cAMP- and cGMP-dependent protein kinase (IC_{50} values of 3–6 μM) (46). In addition, staurosporine, a potent PKC inhibitor (IC_{50} = 10 nM) also effectively inhibits various PTK activities (47). In contrast, a recent report (32) characterizes the highly selective and potent (IC_{50} = 10 nM) effects of the bisindolylmaleimide GF109203X on PKC. Using this inhibitor, we report here that concentrations of GF109203X that fully block PMA-ionomycin–induced secretion from NK cells had no effect on FcR-initiated granule release. This result suggests that although there is a PKC-dependent pathway in NK cells that can modulate granule exocytosis, FcR-initiated secretion is regulated by a PKC-independent pathway.

Recent attention has focused on PI 3-kinase as a separate second messenger system involved in cellular activation (27). This enzyme is activated by a diverse array of receptors with intrinsic or associated PTK activities, including the PDGF, epidermal growth factor, CSF-1, insulin, and IL-2 receptors (for a review, see reference 27). Most recently, TCR and FcR ligation have also been shown to induce PI 3-kinase activation (18–20). Yet, the downstream functional consequences of PI 3-kinase activation remain unclear, again due to difficulties in selectively inhibiting this pathway. However, a recent report (30) highlights the potent inhibitory activity of the fungal metabolite, wortmannin, on PI 3-kinase. Whereas wortmannin was originally characterized as an inhibitor of myosin light chain kinase when used at micromolar concentrations (48), additional studies (30) indicated that at nearly 100-fold lower concentrations, wortmannin selectively and irreversibly inhibits PI 3-kinase. Wortmannin binds to the 100-kD catalytic subunit of PI 3-kinase resulting in inhibition with an IC_{50} of 2 nM (30). We report here that wortmannin similarly inhibits PI 3-kinase activity in NK cells with an IC_{50} of 3 nM and, also inhibits FcR-initiated secretion and cellular cytotoxicity with similar potency. These data suggest that PI 3-kinase activation in NK cells is associated with FcR-initiated granule release and ADCC.

Increasing information implicating potential role for PI 3-kinase in vesicular transport: (*a*) *VPS34*, a homolog of the catalytic subunit of PI 3-kinase, has been identified in the yeast *Saccharomyces cerevisiae* (28). Yeast strains with *VPS34* deletions or containing *VPS34* point mutations exhibit severe defects in the sorting of vacuolar proteins (*b*) during thrombin-stimulated secretion from platelets, PI 3-kinase associates with the membrane cytoskeleton (31); (*c*) mutation of the PI 3-kinase binding site on the PDGF receptor alters endocytic trafficking of this receptor after growth factor binding (29); (*d*) pretreatment of the rat basophilic leukemia cell line, RBL-2H3, with wortmannin inhibits FcεRI-initiated histamine release (30). Our results extend these analyses by demonstrating that ADCC, which requires the formation of a microtubule-organizing center (49) and subsequent granule release at the effector–target interface, appears to require PI 3-kinase activation.

PI 3-kinase activity can be altered by multiple factors. PI 3-kinase associates with receptor proteins and other signaling molecules that contain a tyrosine phosphorylated consensus sequence, thus allosterically modulating its potential activity (36–39, 50–52). Tyrosine and/or serine-threonine phosphorylations may also modulate its enzymatic activity (52–54). In addition, recent reports indicate that p85, the regulatory subunit of PI 3-kinase, associates with the SH3 domain of src family PTK (55, 56) and this interaction can increase PI 3-kinase catalytic activity (57). Because FcR ligation results in the rapid activation of $p56^{lck}$ (9, 58–60), and PTK activation appears to be requisite for granule release and ADCC (61, 62), future work will need to focus on the role of $p56^{lck}$ itself or the proximal substrates of this PTK in coupling FcR stimulation to PI 3-kinase activation.

Finally, data presented here indicate that signaling events regulating granule release after recognition of Ab-coated targets (i.e., ADCC) vs susceptible tumor targets (i.e., direct NK cell–mediated cytotoxicity) are distinct. Early studies on NK cell activation focused on similarities in signaling initiated during these alternative modes of killing, i.e., early PTK activation, inositol phosphate release, and increase in intracellular free Ca^{2+} (4, 6–9, 61–64). However, we previously reported that pretreatment of NK cells with PKC-activating

phorbol esters had differential regulatory effects on these alternative forms of NK cell activation (65). In this report, we extend this analysis by comparatively evaluating the effects of wortmannin and GF109203X on secretion and killing induced by these separate modes of NK cell activation. These data suggest that although FcR-initiated effects on these processes proceed through PI 3-kinase–dependent pathways, direct recognition of susceptible tumor targets initiates granule release and killing through PKC-dependent pathways. These results point out that: (*a*) ADCC and direct natural cytotoxicity are triggered by fundamentally different biochemical events; and (*b*) it may be feasible to differentially modulate the effector functions of NK cells in patients.

The authors thank Christopher J. Dick for his excellent technical assistance and Theresa Lee for her skillful preparation of this manuscript.

This research was supported by the Mayo Foundation and by National Institutes of Health grants CA-47752, CA-52995, and GM-47286.

Address correspondence to Dr. P. J. Leibson, Department of Immunology, Mayo Clinic and Foundation, Rochester, MN 55905.

Received for publication 11 April 1994 and in revised form 13 June 1994.

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