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NATWRAL KILLER CELL AND GRANULOCYTE Fcγ RECEPTOR III (CD16) DIFFER IN MEMBRANE ANCHOR AND SIGNAL TRANSDUCTION[1]

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CD16 is a low affinity FcγR III expressed on granulocytes, macrophages and large granular lymphocytes, the mediators of antibody-dependent cellular cytotoxicity and NK. The occupancy of CD16 by aggregated IgG on large granular lymphocytes induces expression of activation markers, release of inflammatory mediators and triggering of effector functions such as antibody-dependent cellular cytotoxicity. Recently we and others described that CD16 is anchored to the membrane of granulocytes via a phosphatidylinositol glycan moiety. Here we show that the CD16 molecule expressed on NK cells, cultured monocytes, and lung macrophages is not phosphatidylinositol glycan moiety anchored. It is not released with phosphatidylinositol-specific phospholipase C, and after removal of N-linked carbohydrate is 5 to 7 kDa larger than the granulocyte CD16 molecule, strongly suggesting the presence of transmembrane and cytoplasmic protein domains. Redirected killing of hybridoma targets expressing anti-CD16 surface Ig shows that NK cell CD16 is able to trigger killing, whereas granulocyte CD16 is unable to do so. These findings demonstrate that NK cell and granulocyte CD16 have different membrane anchors and indicate that the type of membrane anchor is an important biologic mechanism for regulating the functional capacity of surface receptors.

Surface receptors signal via their membrane and cytoplasmic moieties. Two membrane molecules, LFA-3[3] and neuronal cell adhesion molecule, have isoforms with identical extracellular domains linked to distinctive membrane moieties (1, 2). Alternative mRNA splicing gives rise either to a carboxyl-terminus with both hydrophobic transmembrane and hydrophilic cytoplasmic domains which is a classical polypeptide chain anchor, or to a carboxyl-terminus with a hydrophobic domain which is cleaved off and replaced with a PIG anchor. The functional significance of membrane anchor isoforms is unclear.

The FcγR III (CD16) molecule is expressed on neutrophils, NK cells, liver macrophages (Kupffer cells), and cultured monocytes (3, 4). Although the CD16 molecules on both granulocytes (5, 6) and NK cells (7) have been reported to be PIG-anchored, they have different functional capacities. FcγR III and the lower abundance FcγR II on neutrophils both contribute to ADCC and the respiratory burst, although it appears that polypeptide anchored FcγR II may be the signalling receptor while FcγR III serves to capture ligand (8–11). FcγR III is the only FcγR expressed on NK cells, where it is involved in ADCC (8) and the triggering of lymphokine secretion (3, 12, 13). Here we report cell specific differences in the membrane anchor of granulocyte and NK cell CD16, and correlated differences in their functional capacity.

MATERIALS AND METHODS

Cells. Granulocytes were isolated from blood as described (5). Briefly, blood samples were collected from healthy individuals in the anticoagulant acid citrate dextrose. Leukocyte-rich plasma was separated from E by 6% dextran T-500 (Pharmacia Fine Chemicals, Piscataway, NJ) sedimentation, overlaid on Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) and centrifuged at 1000 × g for 25 min. Granulocytes were recovered from the pellet with some contaminating E which were removed by hypotonic lysis with water for 20 s. NK cells were isolated from plastic and nylon wool nonadherent mononuclear cells obtained from fresh blood or from the buffy coat byproduct of platelet-pheresis by Percoll (Pharmacia) gradient centrifugation as described (14). The cell fractions obtained from Percoll gradients were analyzed by immunofluorescence flow cytometry after staining with anti-CD16 mAb and the $CD16^+$ fractions were used for further experiments. The cells were 50 to 75% $CD16^+$. In some experiments the NK cells were further purified from contaminating $CD3^+$ cells by labelling with CD3 mAb (OKT3) and removing the mAb binding cells by rosetting with magnetic beads (Dynal, Robbins Scientific, Mountain View, CA) coated with F(ab')2 goat anti-mouse IgG and IgM (Tago Inc., Burlingame, CA.) This step resulted in 95% pure LGL as judged by Giemsa-stained cytocentrifuge smears (14). Plastic adherent monocytes were cultured in Teflon beakers with 10% human AB serum (Whittaker M.A. Bioproducts, Walkerville, MD) and 2% FCS (GIBCO, Grand Island, NY) for 15 to 20 days (15). Alveolar macrophages were obtained from bronchoalveolar lavage fluid from a smoker. COS cells were transfected with purified CD16 cDNA in CDM8 using DEAE dextran (7). The cDNA clone kindly provided by B. Seed (Harvard Medical School, Boston, MA). pCD16.2, was distinct from his published CD16 cDNA (15) but known to have an identical transmembrane sequence. Sequencing in our laboratory showed an extracellular domain identical to that described by Peltz et al. (16). Target cell lines used in the cytotoxicity experiments, CLBFcR-gran-1 (IgG2a) and 3G8 (IgG1), were anti-CD16 producing hybridomas and W6/32 was an anti-HLA-A,B (IgG2a) hybridoma. Anti-LFA-1 (TS1/22) and anti-LFA-3 (TS2/9) hybridoma cells were also used. The mAb and hybridomas have been previously referenced (5, 17).

PIPLC treatment of cells and immunofluorescence flow cytometry. PIPLC treatment of cells was carried out as described earlier (5). Briefly, cells (5 to 10 × 10^6) were resuspended in HBSS/HEPES, pH 7.4 containing 1 mg/ml OVA (Sigma) and treated with or without *Bacillus thuringiensis* PIPLC (0.6 mmol/min/ml) for 45 min at 37°C. The cells were then washed, stained with hybridoma culture super-

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[2] To whom correspondence and reprint request should be addressed.
[3] Abbreviations used in this paper: LFA, lymphocyte function-associated Ag; LGL, large granular lymphocytes; ADCC, antibody-dependent cellular cytotoxicity; PIG, phosphatidylinositol glycan; PIPLC, phosphatidylinositol-specific phospholipase C; DAF, decay accelerating factor.

natants followed by FITC conjugated F(ab')2 goat antimouse IgG and IgM (Tago Inc., Burlingame, CA) and subjected to flow cytometry as previously described (17).

Cell surface labelling and SDS-PAGE. Cells were surface labelled with $Na^{125}I$ (New England Nuclear, Boston, MA) using 1, 3, 4, 6-tetrachloro- 3α, 6α-diphenyl glycouril (Pierce Chemical Co., Rockford, IL) (18). Cells were washed and aliquots were treated with PIPLC as described above. The cell pellet was lysed (5) and aliquots of the supernatant and cell lysate were precipitated with CLBFcR-gran1-Sepharose. The immunoprecipitates with or without N-glycanase (Genzyme, Boston, MA) digestion (19) were subjected to SDS 10% PAGE under reducing conditions (20).

Cytotoxicity assays. Granulocytes were preincubated for 18 h in presence of 500 U/ml IFN-γ (Genzyme, Boston, MA). The cytotoxicity of both cell types was tested with a standard ^{51}Cr-release assay (4 h for NK cells, 6 h for granulocytes). Rat mAb M5/11, M5/69, M7/84 (culture supernatants diluted 1/40) reactive with murine myelomas (21, 22) were used in the cytotoxicity assays where indicated to allow granulocyte ADCC via FcγR I/II. In some experiments NK cells were pretreated for 30 min at 4°C with purified 3G8 or CLBFcR-gran1 IgG or F(ab')2 fragment of CLBFcR-gran1. The conjugates formed by effector cells and target cells were measured as described (23).

RESULTS AND DISCUSSION

Effect of PIPLC treatment on the NK cell, monocyte, and granulocyte CD16. We examined whether NK cell and granulocyte CD16 differ in their membrane anchors. Cells were treated with PIPLC followed by quantitation of surface antigens with immunofluorescence flow cytometry. As previously shown (5, 6) CD16 on neutrophils was 75% released by PIPLC (Fig. 1*A*). Controls verified that under the same conditions DAF and LFA-3 were sensitive to PIPLC on neutrophils (Fig. 1*A*). In contrast to neutrophil CD16, CD16 on NK cells was not released by PIPLC (Fig. 1*B*). As controls for releasability of PIG anchored proteins on LGL, we used LFA-3, which is both PIG and polypeptide-anchored, and DAF and CD48 which are PIG anchored (19, 24). Although a previous report found that NK cells are DAF$^-$ (25), we consistently find low levels of DAF expression on LGL. These experiments used both a Percoll-purified LGL preparations with 72% CD16$^+$ cells (Fig. 1), and a CD3$^+$ cell-depleted LGL preparation yielding 95% LGL with 81% CD16$^+$ cells, which were 73, 100, and 100% DAF, CD48 and LFA-3$^+$, respectively. We consistently found DAF and CD48 were largely released from

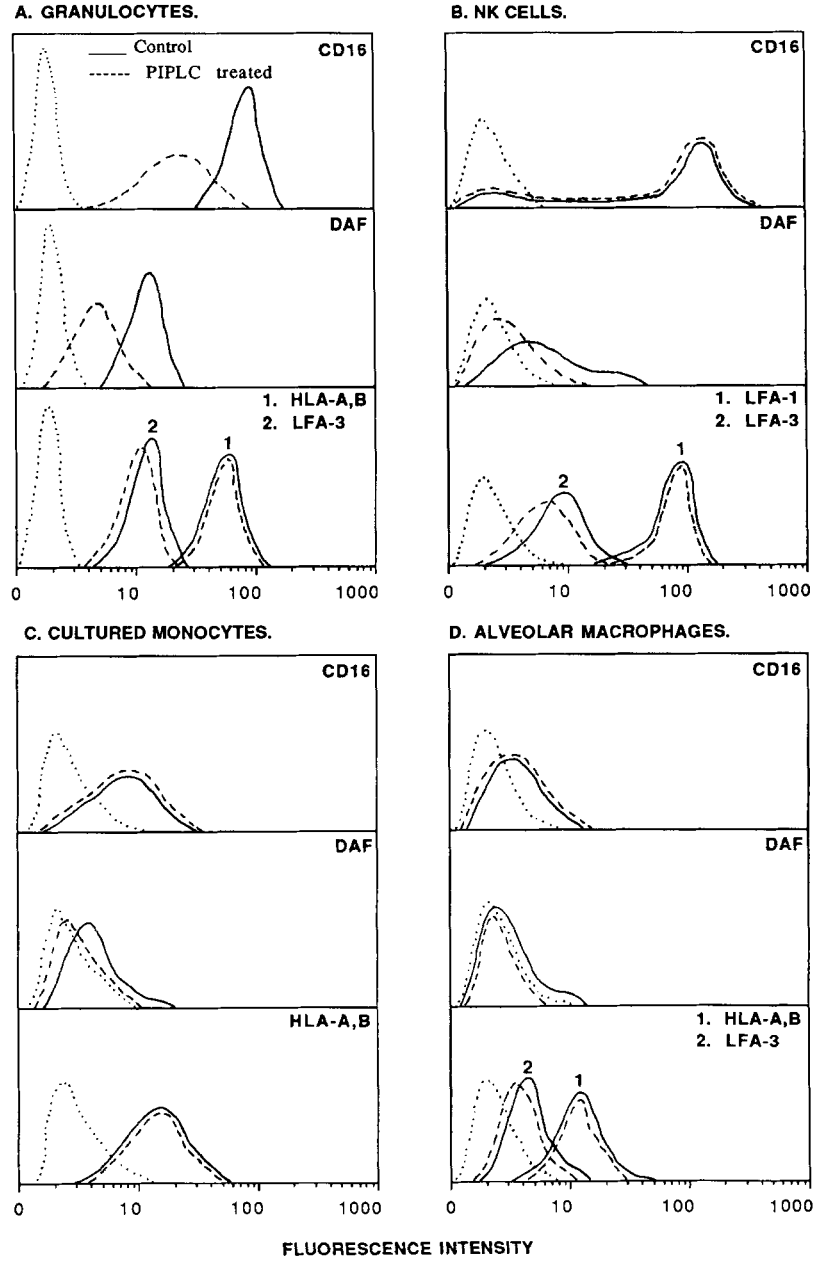

Figure 1. Immunofluorescence flow cytometry. Cells were treated with or without PIPLC and stained with the indicated mAb. Specific mAb staining of cells with (---) and without (——) PIPLC. Control X63 staining with or without PIPLC (identical curves, . . .).

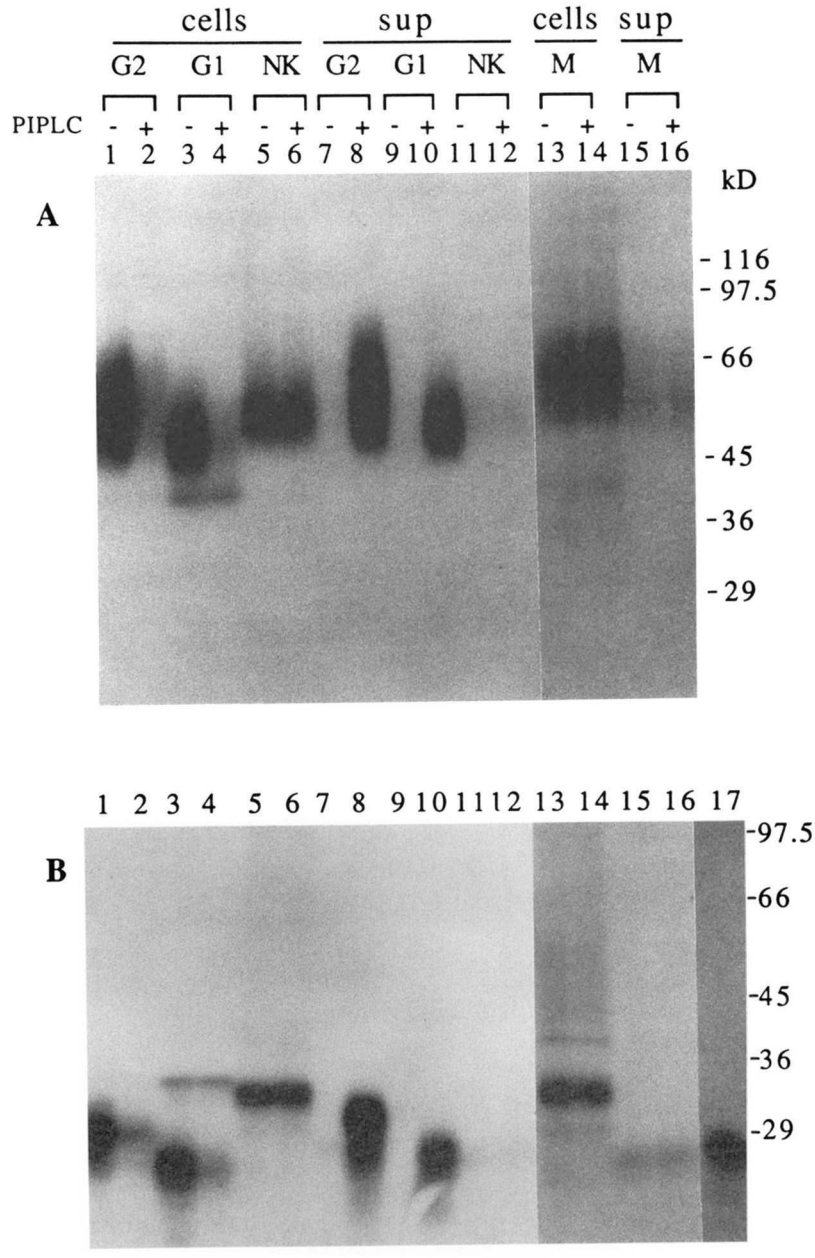

Figure 2. SDS-PAGE analysis of PIPLC treated and N-glycanase digested CD16. *A*, Immunoprecipitation of CD16 from cell lysates and supernatants with and without PIPLC treatment. G2 and G1 represent granulocytes from NA1NA2 heterozygous and NA1 homozygous donors, respectively. NK and M represent NK cells and cultured monocytes respectively. Molecular weight markers are indicated. *B*, N-glycanase digests of CD16 immunoprecipitates. *Lanes 1* to *16* are the same as described in *A* except all the samples were digested with N-glycanase before SDS PAGE. *Lane 17* is PIPLC released and N-glycanase digested CD16 from COS cells transfected with CD16 cDNA.

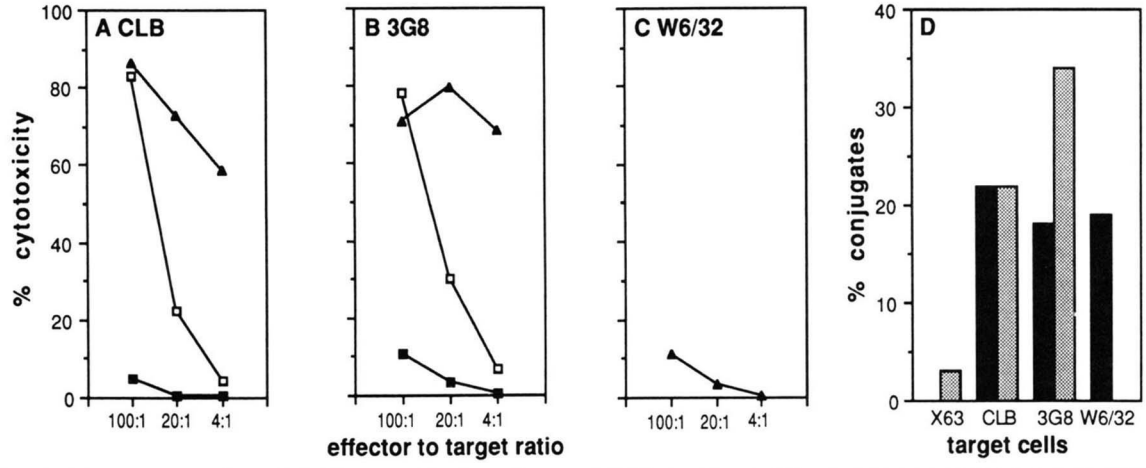

Figure 3. NK cell and granulocyte-mediated lysis and binding of mouse hybridoma targets. NK cells (*closed triangles*), granulocytes in absence (*closed squares*), or presence (*open squares*) of anti-myeloma cell antibodies. Target cells: *A*, CLBFcR-gran1 (CLB); *B*, 3G8 and *C*, W6/32; *D*, Conjugate formation with NK cells, *dark bars*; granulocytes, *shaded bars*. *A*, *B*, and *C* are representative of three experiments and *D* is mean of four experiments.

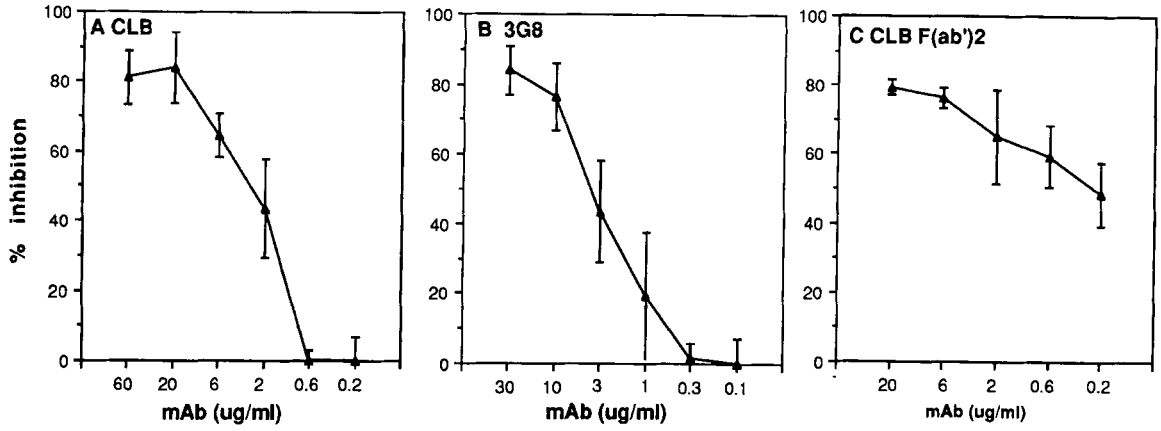

Figure 4. Inhibition of NK-cell mediated lysis of CLBFcR-gran1 hybridoma target cells by CD16 mAb. Purified NK cells were preincubated for 30 min at 4°C with the indicated concentrations of purified CLBFcR gran1 mAb (*A*), 3G8 mAb (*B*), or its F(ab')2 fragment (*C*). ^{51}Cr labelled CLBFcR-gran1 hybridoma cells were added and a standard 4-h cytotoxicity assay was performed with E/T ratio 20:1 and 5:1. *A* and *B* are mean ± SD of four experiments. *C* is mean ± SD of two experiments.

these purified LGL populations and LFA-3 was partially released (Fig. 1 and not shown). Other transmembrane proteins such as HLA-A,B, LFA-1, Mac-1, and FcγR II were unaffected by PIPLC (Fig. 1*B* and not shown). The results were reproducible with NK cell preparations from four different donors.

Blood monocytes are $CD16^-$ but become $CD16^+$ and macrophage-like during culture in vitro for >15 days (15). PIPLC treatment did not have any effect on CD16 found on cultured monocytes and alveolar macrophages although it released proteins like DAF (Fig. 1 *C* to *D*) and CD14 (Mo2) (on cultured monocytes, not shown) that are PIG linked (2). Previous studies on cultured monocytes from paroxysmal nocturnal hemoglobulinuria patients (6) suggested expression of some transmembrane anchored CD16 on at least some monocyte clones; we have extended these findings by showing essentially no expression of a PIG-anchored form of CD16 on cultured monocytes.

The flow cytometry results were confirmed using surface labeled cells. Granulocytes, NK cells, and cultured monocytes were surface iodinated and subjected to PIPLC treatment. CD16 released into the cell supernatant and remaining in the cell pellet was subjected to immunoprecipitation and SDS-PAGE. PIPLC did not affect the amount of cell-associated CD16 on NK cells (Fig 2*A*, *lanes* 5 and 6) and monocytes (Fig 2*A*, *lanes 13* and *14*) and did not release material into the supernatant (Fig. 2*A*, *lanes 11*, *12*, *15*, and *16*) although some material appeared to be spontaneously released from monocytes (Fig 2*A*, *lanes 15* and *16*). In contrast and as previously reported (5, 6), treatment of neutrophils with PIPLC resulted in a decrease in CD16 on cells (Fig 2*A*, *lanes 1* to 4) and appearance of CD16 in the supernatant (Fig 2*A*, *lanes 7* to *10*).

NK cell, monocytes, and granulocyte CD16 differ in their polypeptide backbone. The distinct membrane anchor hypothesis predicts different sizes for granulocyte and NK cell CD16. Molecular size heterogeneity due to N-linked glycosylation (Fig. 2*A*) was removed by N-glycanase digestion (Fig. 2*B*). Neutrophil CD16 exhibits a size polymorphism linked to the NA1 and NA2 CD16 alloantigens (26). The molecular size of the CD16 expressed on NA1 homozygous neutrophils is 26 kD (Fig. 2*B*, *lanes 3* and 4) and on NA1NA2 heterozygous neutrophils is 26 and 28 kD (Fig. 2*B*, *lanes 1* and 2). The molecular size of the CD16 expressed on NK cells (Fig. 2*B*, *lanes 5* and 6) and cultured monocytes (Fig. 2*B*, *lanes 13* and *14*) is 33 kDa, which is 5 to 7 kDa higher than neutrophil CD16. The difference in molecular size observed between CD16 from NK cells and granulocytes after N-glycanase digestion is not due to O-glycosylation; it has recently been reported that granulocyte and NK cell CD16 do not have O-linked glycans (27, 28), and after N-glycanase or endo-F digestion differ in molecular size, which is in complete agreement with our results. Increasing the time of digestion or concentration of N-glycanase did not show any further change in size of CD16 (data not shown). Removal of the diacylglycerol moiety from neutrophil CD16 results in a slight decrease in mobility (Fig. 2*B*, *lanes 8* and *10*), as previously observed for other PIG-anchored proteins (19, 29). We also see small amounts of material of 26 kDa released from monocytes (Fig. 2, *lanes 15* and *16*) and from NK cells after prolonged autoradiogram exposure, but there is as much with or without PIPLC treatment. A contaminating band appearing in some neutrophil immunoprecipitates at 34.5 or 39 kDa with or without deglycosylation respectively (Fig. 2 *A* and *B*, *lanes 3* and 4) was the FcγR II because it could be precleared with CIKM5 mAb (30) (not shown). It has been reported that the CD16 on cultured monocytes migrates as a sharp band in SDS-PAGE, is resistant to N-glycanase and has a molecular weight of 53 kD (15). This is in contrast to our results which show that cultured monocyte CD16 is a broad diffuse band of 65-kDa M_r and sensitive to N-glycanase treatment. The reasons for these differences are not clear.

Resistance of PIG-anchored proteins to PIPLC has been reported (1, 2). The acylation of the 2-OH group of inositol which makes some of the PIG-anchored proteins PIPLC resistant (31), is specific to the cell type rather than to the protein. Thus all known PIG anchored proteins on human E, i.e., LFA-3, DAF, and acetylcholinesterase are PIPLC resistant (2), whereas these same proteins on nucleated cells are all sensitive. Moreover, the molecular size of PIPLC-sensitive and -resistant forms of PIG-anchored LFA-3 are the same (19) indicating that the acylation of the PIG anchor inositol 2-OH group does not influence mobility on SDS-PAGE. Our results show that control PIG anchored proteins on NK cells and monocytes

are susceptible to PIPLC. Furthermore, the molecular size of deglycosylated NK cell and monocyte CD16 is different from that of granulocyte CD16. We conclude that the resistance of CD16 to PIPLC on these cells is not due to a modified, PIPLC-resistant PIG anchor, but due to a polypeptide chain anchor. In contrast to CD16 on neutrophils, CD16 present on NK cells and macrophages is not PIG anchored. The CD16 thus exists in two isoforms with distinct membrane anchor. The results demonstrate cell lineage-specific differences in the membrane proximal region of FcγR III/CD16 molecule.

cDNA cloning of CD16 has revealed two Ig-like extracellular domains with good homology to FcγR II and murine FcγR (7, 16). The CD16 cDNA clones have a hydrophobic transmembrane domain, no cytoplasmic domain, and are PIG anchored when expressed in COS cells (7, 16). Our finding that NK cell CD16 is not PIG anchored suggests that the previously isolated cDNA clone corresponds to the neutrophil rather than to the NK cell isoform, as has recently been confirmed (16). Furthermore, in our hands a CD16 cDNA clone, pCD16.2, which is identical to the published CD16 sequence of Peltz et al. (16) (M. Hibbs, unpublished data), when expressed in COS cells is susceptible to PIPLC and yields material identical in size to that isolated from neutrophils (Fig. 2*B*, *lane 17*).

NK cell and granulocyte CD16 differ in their signaling for cytotoxicity. We compared the functional capacity of the FcγR III isoforms. Neutrophils express both FcγR II and FcγR III, and after stimulation with IFN-γ additionally express FcγR I (11). Killing of antibody opsonized targets cells by neutrophils may involve synergy between these different types of FcγR; to assess FcγR III individually we used anti-FcγR III hybridomas as targets in redirected killing (11). IFN-γ-stimulated neutrophils failed to show specific killing of CD16 hybridomas (Fig. 3 *A* and *B*), as previously reported (11). In contrast, NK cells killed targets bearing CD16 surface Ig very efficiently (Fig. 3 *A* and *B*). Similar results were obtained whether or not the anti-CD16-hybridomas were selected for high expression of cell surface Ig by labeling with FITC-conjugated F(ab')2 goat antimouse IgG and fluorescent cell sorting. Killing was specifically triggered by CD16 on the NK cells, because target hybridomas bearing HLA-A,B mAb (Fig. 3 *C* and *D*), LFA-3 mAb, and LFA-1 mAb (not shown) formed conjugates but were not lysed. Neutrophils formed conjugates with CD16 hybridomas and this was dependent on CD16 as shown by lack of reaction with X63 myeloma cells (Fig. 3*D*), but the conjugation failed to trigger killing. Pretreatment of NK cells with anti-CD16 mAb 3G8 and CLBFcR-gran1 or its F(ab)2 inhibited anti-CD16-hybridoma killing in a dose-dependent manner (Fig. 4) further emphasizing the CD16 requirement in lytic action. In contrast to a recent report questioning the ability of granulocytes to kill tumor target cells (28), we show that IFN-γ-stimulated neutrophils have the machinery for killing because they could kill the target cells after opsonization with mAb (Fig. 3 *A* and *B*). Granulocytes have been previously shown to be functional in redirected killing of hybridomas via the FcγR II and FcγR I (11) which apparently trigger the ADCC seen in our experiments. Thus PIG-anchored neutrophil CD16 does not trigger killing of nucleated target cells whereas the protein anchored CD16 on NK cells is a potent trigger of cytolytic function.

Significance of distinct membrane anchored isoforms of CD16. Neutrophils are involved in clearance of immune complexes from the circulation (3). mAb to either FcγR III or FcγR II alone can inhibit neutrophil functions suggesting that both FcγR may synergize in interaction with multivalent immune complexes. Previously it was suggested that the PIG anchor on the neutrophil FcγR III made this a weakly signaling receptor (5), based on functional comparisons to the protein anchored FcγR I and FcγR II on the same cell (11).

Immune complexes must be removed from the circulation without causing disseminated neutrophil activation; otherwise severe immunopathologic reactions ensue. The PIG anchor may allow efficient ligand binding and internalization to be coupled with relatively weak signaling. The high density of FcγR III and its predicted fast mobility in the membrane bilayer may suit it for capture of immune complexes. We and others (28, 33) have shown that neutrophil FcγR III does not mediate ADCC of nucleated targets. LGL are the cells in blood which mediate natural killing and ADCC (32). CD16 is the only FcγR on NK cells and as shown here and elsewhere (28) mediates ADCC by these cells. Presence of an FcγR which can trigger target cell killing is of importance for the NK cell to mediate its function. Therefore expression of a CD16 with a protein anchor which can trigger cytotoxicity of NK cells and a CD16 with PIG anchor which can enhance capturing immune complexes on neutrophils but does not trigger killing may be of great physiologic significance. Comparisons between neutrophil PIG-anchored FcγR III and NK cell protein-anchored FcγR III now further emphasize this point, and demonstrate that PIG and protein-anchored isoforms of membrane anchored proteins may have profound consequences for biologic function.

Note added in proof. E. Ueda, T. Kinoshita, J. Nojima, K. Inoue, and T. Kitani (1989. *J. Immunol. 143:1274*), and J. C. Edberg, P. B. Redecha, J. E. Salmon, and R. P. Kimberly (1989. *J. Immunol. 143:1642*) also find neutrophil and NK cell CD16 have distinct membrane anchors. J. V. Ravetch and B. L. Perussin (1989. *J. Exp. Med. 170:481*) and B. J. Scallon, E. Scigliano, V. H. Freedman, M. C. Miedel, Y. E. Pan, J. C. Unkeless, and J. P. Koch (1989. *Proc. Natl. Acad. Sci. USA 86:5079*) show neutrophil and NK cell CD16 are encoded by highly homologous genes that differ by only 4 or 6 amino acid substitutions (depending on the neutrophil gene NA type) and presence of a cytoplasmic domain of 21 amino acids in the NK cell isoform, in agreement with results presented here.

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