Preferential Expression of Human FcγRIII$_{PMN}$ (CD16) in Paroxysmal Nocturnal Hemoglobinuria

Discordant Expression of Glycosyl Phosphatidylinositol-linked Proteins

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Abstract

The isoform of FcγRIII (CD16) expressed on PMN has a GPI membrane anchor, and in paroxysmal nocturnal hemoglobinuria (PNH) there is a deficiency in FcγRIII expression on PMN. Contrary to expectation, however, CD16 expression is preserved (albeit at reduced levels) in all affected PNH PMN that completely lack the GPI-anchored proteins DAF (CD55) and CD59. FcγRIII negative PMN are not observed in any of the six PNH patients examined in this study. Analysis of the molecular weight of both glycosylated and deglycosylated FcγRIII from PMN with reduced FcγRIII expression indicates no variations in size relative to normal donor FcγRIII$_{PMN}$. Indeed, the FcγRIII expressed at intermediate levels is phosphotidylinositol-specific phospholipase C (PI-PLC)-sensitive. Thus, there is no evidence suggestive of expression of a transmembrane isoform and all data indicate that FcγRIII$_{PMN}$ on affected cells in PNH is a GPI-linked isoform. With FcγRIII$_{PMN}$ expression preserved at reduced levels on affected cells in PNH, PMN from PNH patients retain the capacity to internalize the FcγRIII$_{PMN}$-specific probe E-ConA (at reduced levels) as well as IgG-opsonized erythrocytes. Reduced expression of GPI-anchored molecules on PNH PMN is not restricted to FcγRIII$_{PMN}$ since intermediate levels of CD59 were also observed in the PNH PMN that were decay-accelerating factor (DAF)-negative and FcγRIII$_{PMN}$ intermediate. In addition, discordant expression of GPI-linked molecules in individual cells is not restricted to PMN since DAF$^+$/CD14$^-$ monocytes were observed in one PNH patient. These data suggest that, when analyzed on an individual cell level, the GPI anchor defect in PNH is not absolute and must involve either a hierarchy of access of different protein molecules to available GPI anchors, distinct anchor biochemistries for the different proteins, or differential regulation of protein-anchor assembly. (*J. Clin. Invest.* 1991. 87:58–67.) Key words: glycosyl-phosphatidylinositol • paroxysmal nocturnal hemoglobinuria • Fcγ receptor • decay-accelerating factor • CD59

Introduction

Paroxysmal nocturnal hemoglobinuria (PNH)[1] is an acquired hemolytic anemia of clonal origins in which erythrocytes are sensitive to complement-mediated lysis (1, 2). In PNH, it has been proposed that there is a generalized deficiency in the ability of affected cells to link proteins to the cell surface membrane with a glycosyl phosphatidylinositol (GPI) anchor (2–6). Since many complement regulatory proteins that protect cells against complement lysis are linked to the membrane by GPI anchors (5–8), a deficiency in these proteins is thought to play a central role in the pathogenesis of PNH (2, 9–11). Support for a generalized, clonal defect of GPI anchoring in PNH comes from studies showing concordant deficiencies of multiple GPI-linked proteins (12–14).

Recent work on the structure of human FcγRIII (CD16) has demonstrated the presence of two receptor isoforms, a GPI-linked and a transmembrane form, which are encoded for by distinct, yet highly homologous, genes (15–17). The expression of these genes is highly regulated in that the GPI-linked gene product is found in PMN and the transmembrane gene product is found in natural killer (NK) cells and macrophages (Mϕ) (15–23). Patients with PNH have been shown to have normal levels of FcγRIII$_{NK}$ and FcγRIII$_{Mϕ}$ (21–23) but are deficient in FcγRIII$_{PMN}$ (18, 19), consistent with the neutrophil isoform having a GPI anchor. Since CD16 participates in the handling of immune complexes and certain bacteria (24–27), a generalized deficiency in FcγRIII$_{PMN}$ might lead to significant impairment of host defenses. However, the absence of prominent immune complex–mediated disease, frequent bacterial infections, and recurrent viral infections (1, 28, 29) suggests either that CD16 is not essential for protection of the host or that it is not totally deficient on PNH PMN.

To address the role of CD16 expression in PNH, we examined both myeloid and lymphoid cells for CD16 in conjunction with other GPI-anchored molecules. As expected, FcγRIII$_{NK}$ expression was normal on both DAF$^+$ and DAF$^-$ NK cells, consistent with the expression of only the transmembrane receptor isoform. However, contrary to expectation, we found preserved expression of CD16 (albeit reduced) in DAF$^-$ and

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1. *Abbreviations used in this paper:* DAF, decay accelerating factor; FcγR, receptor for the Fc region of IgG; FcγRIII$_{NK}$, receptor for the Fc region of IgG expressed on NK cells; FcγRIII$_{PMN}$, the 50–80 kD receptor for the Fc region of IgG expressed on polymorphonuclear leukocytes; GPI, glycosyl-phosphatidylinositol; Mϕ, macrophages; NK, natural killer cell; PE, phycoerythrin; PI-PLC, phosphatidylinositol-specific phospholipase C; PNH, paroxysmal nocturnal hemoglobinuria.

$CD59^-$ PMN. The molecular weight of CD16 expressed on the DAF^- and $CD59^-$ PMN was identical to normal nontransmembrane, GPI-anchored control $Fc\gamma RIII_{PMN}$ (both glycosylated and deglycosylated) and was PI-PLC sensitive. Thus, $Fc\gamma RIII_{PMN}$ in affected PNH PMN has a GPI-anchor form rather than (*a*) a transmembrane form like the same receptor co-transfected with γ chain in anchor-deficient L cells (17, 30), or (*b*) both GPI and transmembrane anchor forms like LFA-3 and N-CAM (reviewed in reference 31). These data indicate that, when analyzed on a clonal level, the GPI anchor defect in PNH is not absolute and must involve either a hierarchy of access of different protein molecules to available GPI anchors, distinct anchor biochemistries for the different proteins, or differential regulation of protein-anchor assembly. Since these PMN retain the capacity to internalize IgG-opsonized erythrocytes, albeit with a reduced capacity to internalize the $Fc\gamma RIII_{PMN}$-specific probe E-ConA, host defenses of patients with PNH may be protected by the relative preservation of $Fc\gamma RIII$ (CD16) expression.

Methods

Reagents. HBSS and FCS were from Gibco Laboratories (Grand Island, NY). FCS was heat inactivated at 56°C for 60 min before use. FITC-conjugated goat anti-mouse $F(ab')_2$ was purchased from Organon Teknica (Malvern, PA). Phycoerythrin (PE)-conjugated goat anti-mouse $F(ab')_2$ was purchased from Tago Immunochemicals (Burlingame, CA). Both conjugated reagents were absorbed against human mononuclear cells (MNC) before use.

MAbs used in this study included 3G8, a mouse (m) IgG1 specific for $Fc\gamma RIII$, provided by Dr. J. Unkeless, Mt. Sinai Medical Center, New York, NY (32); CLB FcR-gran1 and CLB gran11, both mIgG2a specific for $Fc\gamma RIII$, provided by Dr. P. Tetteroo, Central Laboratory of the Netherlands Red Cross, Amsterdam (33); IV.3, a mIgG2b specific for $Fc\gamma RII$ expressed on neutrophils, monocytes, and other cell types, provided by Dr. P. Guyre, Dartmouth Medical School, Hanover, NH (34); IA10, a mIgG2a specific for DAF (2), provided by Dr. M. E. Medof, Case-Western Reserve Medical School, Cleveland, OH and Dr. M. Davitz, New York University Medical Center, New York, NY; MEM-43, a mIgG2a specific for CD59, provided by Dr. I. Stefanová, Czechoslovak Academy of Sciences, Prague (35). Anti-CD14 MAb MO2-FITC (mIgM), anti-CD3 MAb T3 (IgG1), and anti-CD20 MAb B1 (IgG2a) were purchased from Coulter Immunology (Hialeah, FL). Anti-CD14 MAb LeuM3-PE (mIgG2b) was purchased from Becton-Dickinson & Co. (Mountain View, CA). Isotype controls (mIgG1 [MOPC21] and mIgG2a [UPC10]) were purchased from Sigma Chemical Co. (St. Louis, MO). A biotin conjugate of MAb 3G8 was prepared with NHS-biotin following the manufacturers recommendations (Pierce Chemical Co., Rockford, IL) and a FITC conjugate of MAb IA10 was prepared using standard techniques. Avidin-FITC (Vector Laboratories, Burlingame, CA) was used for development of mAb 3G8-biotin staining. Finally, phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* was kindly provided by Dr. M. Low, Columbia University, New York, NY (3, 4, 31).

Patients and controls. Peripheral blood was collected from PNH patients and from controls in heparinized tubes. All six patients had an established clinical diagnosis of PNH with positive acidified-serum lysis (Ham) and sucrose hemolysis tests. Peripheral blood from healthy controls was collected in parallel with each PNH patient and processed identically. Each PNH patient was studied on at least two separate occasions. All donors were typed for the $Fc\gamma RIII_{PMN}$-specific NA polymorphism as previously described (20, 23, 36).

Cell preparations. Fresh anticoagulated human peripheral blood was separated by centrifugation through a discontinuous two-step Ficoll-Hypaque gradient (37). PMN were isolated from the lower interface and washed with HBSS. Contaminating erythrocytes were lysed with hypotonic saline (0.2% NaCl) for 20 s followed by 0.16% NaCl and a final wash with HBSS. Mixed MNC were isolated from the upper Ficoll-Hypaque interface and washed with HBSS. After final washes, all cells were resuspended to 6×10^6 cells/ml before immunofluorescent staining.

Immunofluorescence flow cytometry (37). An aliquot of cells (50 μl at 6×10^6/mL) were incubated with saturating amounts of specific MAb or isotype controls for 30 min at 4°C. After two washes with cold PBS containing 0.1% FCS, unconjugated MAb were incubated with fluorochrome-conjugated $F(ab')_2$ anti-mouse IgG for 30 min at 4°C. Subsequently, cells were again washed twice with cold PBS/0.1% FCS. For two-color flow cytometry, stained cells were incubated with mIgG1 and mIgG2a, each at 10 μg/ml, for 30 min at 4°C to saturate any remaining mIgG binding sites on the fluorochrome-conjugated $F(ab')_2$ anti-mouse IgG. After two more washes with cold PBS/0.1% FCS, these cells were stained with the second directly conjugated (or biotinylated) MAb as above. After the final washes, all cells were maintained in the dark at 4°C until analysis. Greater sensitivity was found with the PE-conjugated, rather than the FITC-conjugated, second antibody reagent. In two-color experiments, a biotin conjugate of MAb 3G8 was also used followed by FITC-conjugated avidin.

Cell-associated immunofluorescence was quantitated within 1–2 h after staining on a Cytofluorograf IIs using a three and one half decade logarithmic amplifier and a 2151 computer (Becton-Dickinson Immunocytometry Systems, Westwood, MA). Exciting light for both FITC and PE at 488 nm was provided by an argon-ion laser at an incident power of 40 mW. Before each experiment, the instrument was calibrated with FITC-conjugated calf thymus nuclei (Fluorotrol-GF, Becton-Dickinson & Co.) and with PE microbead standards (Flow Cytometry Standards Corp., Research Triangle Park, NC). For two-color flow cytometry, cells were run both compensated and uncompensated for spectral overlap of PE fluorescence into the FITC detector and for FITC fluorescence into the PE detector. In the uncompensated mode, maximal sensitivity for both fluorochromes is maintained although the rectilinear format of two-color displays is lost. However, with nonrectangular gating based on bit-mapped graphics on the 2151, the ability to gate individually on each cell population in uncompensated analysis was not compromised.

All MAb immunofluorescence samples were prepared both as single-color and two-color samples and analyzed accordingly. PMN were identified by characteristic forward and right angle light scattering and by MAb IV.3 fluorescence. Blood monocytes were identified by characteristic forward and right angle light scattering and by characteristic MAb IV.3 fluorescence (38). Cells in the monocyte light scatter gate were negative for the T cell and B cell markers T3 and B1. NK cells were also identified by characteristic forward and right angle light scatter and by MAb 3G8 immunofluorescence. To examine the clonality of affected cells in PNH (23), we used two-color flow cytometry to measure the simultaneous expression of GPI-linked proteins on defined cell populations. Under all conditions, isotype controls for each color in the presence of the appropriate positive MAb in the second color were analyzed in order to interpret the positivity of the two-color samples (23).

MAb competition and epitope specificity for MAb 3G8 staining on PNH PMN. Previous work has demonstrated that the immunofluorescence associated with MAb 3G8 on normal PMN represents specific epitope-directed binding (32, 37). To confirm the specificity of both the intermediate and the normal levels of MAb 3G8 staining on PNH PMN (see Results), MAb competition experiments were performed. PMN from both normal and PNH patients were incubated with an excess of MAb CLB FcR-gran1, a MAb that binds to the ligand binding site of $Fc\gamma RIII$ like 3G8 (33), at 4°C for 20 min and then stained with MAb 3G8-biotin followed by avidin-FITC.

PI-PLC sensitivity of CD16 on PNH PMN. The relative sensitivity to PI-PLC of both normal and intermediately expressed levels of CD16 was determined by digestion with enzyme derived from *B. thuringiensis*. Incubation with PI-PLC leads to partial removal of a large number of different GPI-anchored molecules, each with a characteristic degree

of sensitivity (3, 31, 39). For PI-PLC digestion experiments, washed PMN in PBS were treated with 0.39 μmol min/ml PI-PLC at 37°C for 1 h (20, 23) followed by staining with MAb 3G8. This concentration of PI-PLC maximally cleaves FcγRIII from the surface of PMN. Controls included mIgG1 for nonspecific staining, MAb IV.3 (which recognizes the transmembrane protein FcγRII) as a PI-PLC–resistant marker and MAb IA10 (anti-DAF) as a PI-PLC–sensitive marker.

Enzyme digestion and SDS-PAGE. Immunoprecipitates (still bound to the MAb-Sepharose) were resuspended in nonreduced SDS-PAGE buffer and were loaded onto 10% SDS-PAGE gels (20, 23). For removal of N-linked oligosaccharides, the MAb 3G8 immunoprecipitate (bound to the MAb-Sepharose) was incubated with 0.5% SDS at 56°C for 30 min and the supernatant was harvested. Peptide/N-glycosidase F (*N*-glycanase) (Genzyme Corp., Boston, MA) was added and the digestion was then performed in the presence or absence of 2-ME according to the vendor's recommendations (Genzyme Corp.) with 10–40 U/ml of enzyme for 18 or 40 h at 37°C followed by SDS-PAGE (20, 23, 37).

Assays for phagocytosis by PMN. Quantitation of the capacity of PMN to ingest target particles was performed as previously described (27). Antibody-coated erythrocytes (EA) were prepared with rabbit IgG anti–bovine erythrocyte antibodies (27) and ConA–treated erythrocytes (E-Con A) were prepared by a modification of the method of Goldman and Cooper (40) and Salmon and Kimberly (41). To assess internalization of target particles, PMN were combined with EA or E-Con A at a ratio of 1:50. The PMN-erythrocyte mixtures were centrifuged at 44 *g* for 3 min and then incubated at 37°C for 30 min. After lysis of noninternalized erythrocytes with ammonium chloride, phagocytosis was quantitated by light microscopy. Each experiment was performed in a paired fashion with a PNH and a normal donor studied simultaneously, using EA and E-Con A as target particles. At least 500 cells per slide were counted in duplicate without knowledge of the donor identity. Three of the PNH patients were studied once. Each of the remaining three patients were studied twice over a period of at least 2 mo. The data are expressed as percent phagocytosis (%P; percentage of PMN with one or more internalized targets) and phagocytic index (PI; the number of ingested particles per 100 PMN).

Data analysis. Fluorescence intensity was acquired in log fluorescence units and displayed graphically on a superimposed linear scale by the 2151 computer. For single-color intensity, the three and one half decade range of fluorescence intensity is represented as 1–200 linear units (19 U = twofold difference in intensity). The same range is represented as 1–100 linear units in two-color cytograms (9 U = twofold difference in intensity). Normal levels of $Fc\gamma RIII_{PMN}$ and $Fc\gamma RIII_{NK}$ expression were established based on population studies of 49 normal donors (20, 36). Normal concurrent controls for the PNH patients fell within the established range for normal receptor expression.

Statistical comparisons of epitope expression by normals and PNH patients were performed with the paired *t*-test. For statistical analysis of phagocytosis, only the first assay on each PNH patient was used for paired analysis with normal controls (paired *t*-test). A probability of 0.05 was used to reject the null hypothesis that there is no difference between the groups.

Results

FcγRIII expression in PNH. Expression of the GPI-linked protein CD16 on PMN from six PNH patients was examined with the anti-FcγRIII MAb 3G8, and two patterns of FcγRIII expression were observed. One group of four PNH patients displayed two distinct populations of $CD16^+$ PMN (Fig. 1 *A*). The first population of cells, representing 11–88% of the total PMN, showed a high density of $Fc\gamma RIII_{PMN}$ indistinguishable from normal (Fig. 1 *C*), while the second population showed an intermediate level of $Fc\gamma RIII_{PMN}$ (Fig. 1 *A*). The other group of PNH patients ($n = 2$) expressed uniformly intermediate levels

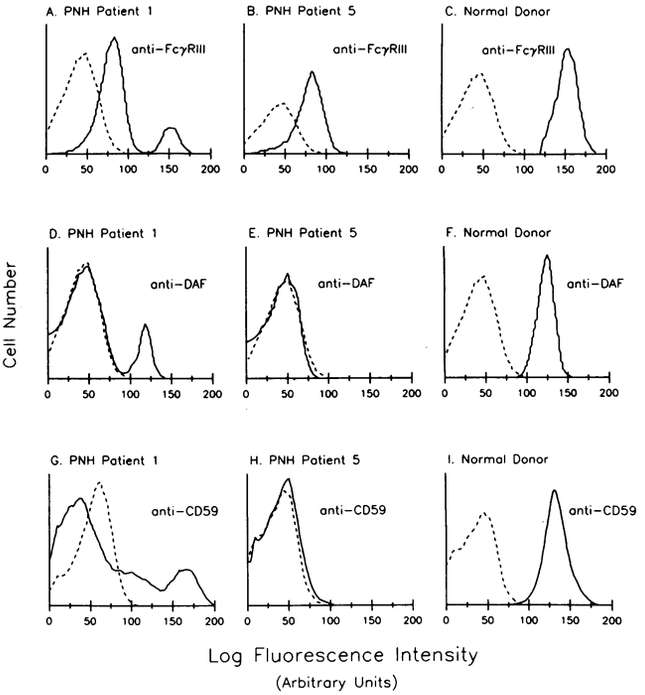

Figure 1. Expression of GPI-linked proteins on PMN from PNH patients and normal controls. Fresh PMN from PNH patients 1 (*A, D, G*) and 5 (*B, E, H*) and from a healthy control (*C, F, I*) were incubated with anti-FcγRIII MAb 3G8 (*A–C*), anti-DAF MAb IA10 (*D–F*), anti-CD59 MAb MEM-43 (*G–I*) or control mIgG1 or mIgG2a (– –), stained with PE-conjugated goat anti–mouse IgG and analyzed by flow cytometry. The bimodal $Fc\gamma RIII_{PMN}$, DAF, and CD59 (*A, D, G*) expression was characteristic of PNH patients 1–4, while the remaining two patients (5 and 6) were $Fc\gamma RIII_{PMN}$ intermediate and DAF^- and $CD59^-$ (*B, E, H*). The three and one half decade range of fluorescence intensity in this figure, and all in other single-color histograms, is represented as 1–200 linear units (19 U = twofold difference in intensity).

of $Fc\gamma RIII_{PMN}$ (Fig. 1 *B*) with fluorescence intensity equal to the intermediate peak observed on the bimodal PNH PMN. This intermediate level of expression, (mean fluorescence intensity, 90.5±15.0) was clearly below normal $Fc\gamma RIII_{PMN}$ (176.4±17.2) and above background nonspecific staining observed with MAb isotype controls (30.0±8.5) (Fig. 1, *A* and *B*). We also examined NK cells for the expression of $Fc\gamma RIII_{NK}$. As expected, NK cells from PNH patients with both bimodal $Fc\gamma RIII_{PMN}$ and uniform intermediate $Fc\gamma RIII_{PMN}$ expressed normal epitope density of the transmembrane $Fc\gamma RIII_{NK}$ (mean fluorescence channel for all PNH patients: 133±14, normal donors: 131±11 with the PE-conjugated anti–mouse IgG).

Expression of other GPI-anchored molecules on PNH PMN. Since we could not find any $Fc\gamma RIII^-_{PMN}$ cells, we examined the same PNH PMN for the expression of DAF and CD59, both GPI-linked proteins (5, 6, 8, 35), to determine whether partial reduction of $Fc\gamma RIII_{PMN}$ was unique to this glycolipid anchored molecule. PNH patients with bimodal $Fc\gamma RIII_{PMN}$ also expressed DAF and CD59 in a bimodal manner (Fig. 1, *D* and *G*). However, while the DAF and CD59 "bright" populations were equal in intensity to normal PMN (Fig. 1, *F* and *I*), the second population was similar to the isotype control MAb. Similarly, PMN from patients who ex-

pressed uniformly intermediate levels of FcγRIII were uniformly DAF⁻ and CD59⁻ (Fig. 1, *E* and *H*).

Further characterization of the FcγRIII$_{PMN}$ intermediate phenotype. Since several investigators have observed complete anchor deficiency in clonally affected cells (2, 9–14, 42–44), we examined the possibility that the specificity of the intermediate MAb 3G8 reactivity might represent nonspecific binding to PNH PMN. In a series of competition experiments both normal PMN and PNH PMN, expressing intermediate levels of FcγRIII, were preincubated with another anti-FcγRIII MAb, CLB FcR-gran1. This MAb, like MAb 3G8, binds to the ligand binding site of FcγRIII. Preincubation with MAb CLB FcR-gran1 completely blocked MAb 3G8-biotin binding and reduced immunofluorescence to the level of background avidin-FITC staining in both the normal (results not shown) and PNH PMN (Fig. 2 *A*), indicating that the intermediate level of MAb 3G8 reactivity represents epitope-specific binding.

To investigate the molecular mechanism leading to the intermediate phenotype of FcγRIII$_{PMN}$, we considered recent work on the structure of FcγRIII$_{PMN}$. The translated FcγRIII$_{PMN}$ protein is efficiently processed by proteolytic cleavage of the transmembrane domain before GPI anchor attachment (4, 17, 30, 45). However, in L cells, which lack the ability to form GPI anchors, a PI-PLC resistant form of FcγRIII$_{PMN}$ can be expressed through a noncovalent interaction with the co-transfected γ chain of FcεRI, presumably with the transmembrane domain of FcγRIII$_{PMN}$ (17, 30, 45). The protein core of this uncleaved, transmembrane form of FcγRIII$_{PMN}$ is ~ 3 kD larger than the mature GPI-anchored form. Thus, we considered the possibility that the GPI anchor deficiency in affected PNH cells was absolute, but that a different molecular form of FcγRIII$_{PMN}$, analogous to L cells, might be expressed in cells affected by the PNH lesion. To address this possibility, we immunoprecipitated FcγRIII from PMN from PNH patients with varying levels of intermediate FcγRIII$_{PMN}$. The two patients at the extremes of bimodal FcγRIII$_{PMN}$ expression were deemed most suitable for these experiments; both donors were NA1 homozygotes by conventional serotyping and by quantitative reactivity with the NA1-specific MAb CLB Gran11 (20, 33, 36). Analysis of MAb 3G8 immunoprecipitates demonstrated that FcγRIII$_{PMN}$ from PNH patients 1 and 3 was 50–65 kD (Fig. 3, *B* and *C*) while NA 1/2 heterozygous normal donor's FcγRIII$_{PMN}$ was 50–80 kD (Fig. 3 *A*) and an NA1 homozygous normal donor's FcγRIII$_{PMN}$ was 50–65 kD (Fig. 3 *D*). Thus, the size of the fully glycosylated FcγRIII$_{PMN}$ from these PNH patients is identical to the NA1 allele of FcγRIII$_{PMN}$ (20, 33). Upon enzymatic removal of N-linked oligosaccharides, FcγRIII$_{PMN}$ from PNH patients 1 and 3 was resolved to a single band of 19 kD (unreduced), which is identical in size to the NA1 allele from normal donors (Fig. 3). The absence of a second band larger than the NA1 allele in the deglycosylated immunoprecipitates from the PNH patients indicates that an unprocessed transmembrane form of FcγRIII$_{PMN}$ (which would be 3 kD larger in molecular weight [17] and perhaps associated with an accessory chain) does not provide the molecular mechanism for intermediate expression of FcγRIII$_{PMN}$ in PNH.

When expressed in L cells in conjunction with γ chain, the FcγRIII$_{PMN}$ form of the receptor is PI-PLC resistant (17, 30, 45). To obtain further insight into the molecular mechanism of the FcγRIII$_{PMN}$ intermediate phenotype and to determine if affected cells could express a GPI anchor, we analyzed the membrane linkage of the intermediate FcγRIII$_{PMN}$ by looking for differential sensitivity of the normal and the intermediate FcγRIII$_{PMN}$ peaks to PI-PLC digestion. Previous studies of enzymatic digestion of FcγRIII$_{PMN}$ with PI-PLC have shown that PI-PLC reduces FcγRIII$_{PMN}$ epitope density but does not result in complete loss of epitope from the cell surface (15, 16, 18–21) (Fig. 2 *B*). Increasing the PI-PLC concentration fourfold and lengthening the incubation period does not enhance removal (data not shown). Incomplete removal of other GPI-anchored molecules from the surface of both erythrocytes and leukocytes is well documented (3, 31, 39), and has been shown to be due to acylation of the inositol head group (46–48). Although re-expression of CD16 epitope could provide an alternative explanation, this does not occur without specific cell activation (Tosi, M., personal communication).

Treatment of PMN with bi-modal expression of FcγRIII with PI-PLC resulted in a reduction of > 50% in both the normal peak and the intermediate peak based on analysis of fluorescence intensity of the individual peaks (Fig. 2 *C*). Likewise, PI-PLC digestion of PMN from a PNH patient with only intermediate FcγRIII$_{PMN}$ resulted in a decrease of ~ 50% in fluorescence intensity (Fig. 2 *D*). Thus, the FcγRIII$_{PMN}$ expressed in those cells with an intermediate level of the receptor is sensitive to PI-PLC like normal FcγRIII$_{PMN}$. Based on analysis of the molecular weight of both glycosylated and deglycosylated

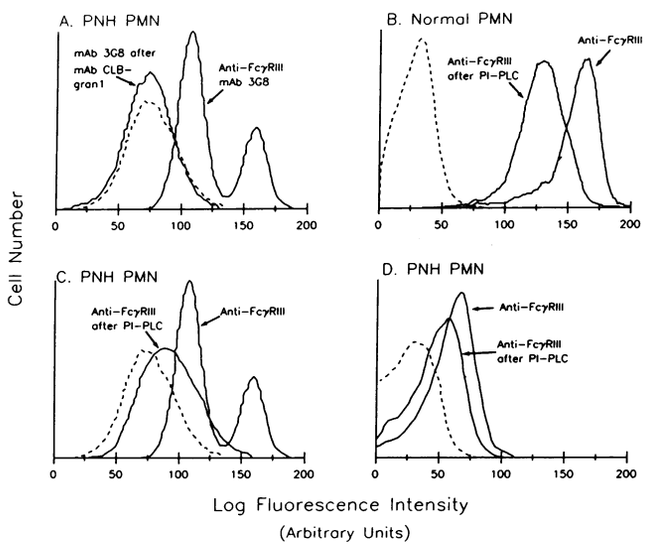

Figure 2. Intermediate FcγRIII$_{PMN}$: epitope specificity and PI-PLC sensitivity. (*A*) To establish the epitope specificity of the MAb 3G8 staining, fresh PMN from PNH patient 2 were preincubated with anti-CD16 MAb CLB-gran1 for 15 min at 4°C. The cells were then incubated with biotinylated mAb 3G8, stained with avidin-FITC or avidin-FITC alone (– –), and analyzed by flow cytometry. Preincubation of the PMN with isotype controls did not change the binding of biotinylated MAb 3G8. The three and one half decade range of fluorescence intensity is represented as 1–200 linear units (19 U = twofold difference in intensity). (*B*) PI-PLC digestion does not lead to complete removal of GPI-linked proteins. Normal donor PMN were treated with PI-PLC for 60 min at 37°C and prepared as above for analysis by flow cytometry. (*C* and *D*). To establish the anchor form of intermediate FcγRIII$_{PMN}$, PMN from PNH patient 2 (with bi-modal FcγRIII$_{PMN}$) (*C*) and from PNH patient 5 (with uni-modal FcγRIII$_{PMN}$) (*D*) were incubated with PI-PLC for 60 min at 37°C and prepared as above for analysis by flow cytometry.

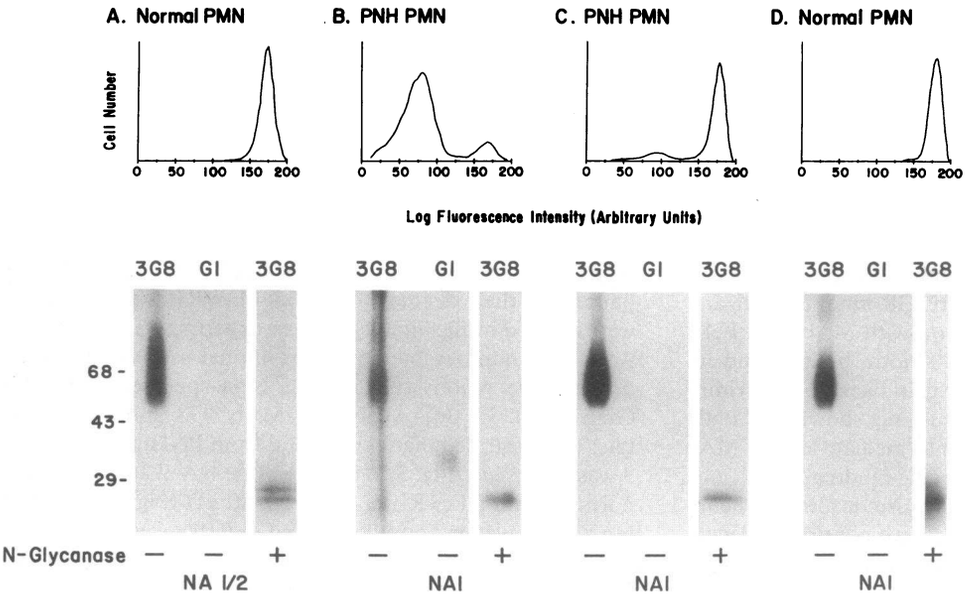

Figure 3. Immunoprecipitation of $Fc\gamma RIII_{PMN}$ from PNH PMN. PMN were ^{125}I labeled, lysed with 1% NP-40 with protease inhibitors (see Methods), immunoprecipitated with MAb 3G8 (or mIgG1) and goat anti-mouse IgG–coated Sepharose, and analyzed by SDS-PAGE. For N-glycanase digestion, immunoprecipitated receptor was first denatured with 0.5% SDS followed by digestion according to the manufacturer's instructions (see Methods) and SDS-PAGE. Both PNH PMN N-glycanase lanes were run on the same gel and adjacent to normal donor PMN N-glycanase lanes. In all cases, the PNH band lined up with the band from the normal donor's NA1 allele. The MAb 3G8 immunofluorescence pattern is shown above the immunoprecipitation data for each individual examined. (*A*) Normal NA 1/2 donor. (*B*) PNH patient 1 (NA1) with 89% affected cells. (*C*) PNH patient 3 (NA1) with 12% affected cells. (*D*) Normal NA1 donor.

$Fc\gamma RIII_{PMN}$ and of PI-PLC sensitivity, the intermediate $Fc\gamma RIII_{PMN}$ phenotype is a GPI-linked form of the molecule, not a transmembrane form.

Discordant expression of FcγRIII and DAF/CD59 on PMN. The absence of an uncleaved transmembrane form of FcγRIII as a unique molecular mechanism for the intermediate expression of $Fc\gamma RIII_{PMN}$ in these PNH cells prompted more detailed study of concordant or discordant expression of various GPI-anchored molecules on a single cell level. Implicit in the dissociation of expression of different GPI-anchored proteins is the potential for different anchor biochemistries and for clonally distinct GPI anchor deficiencies. Indeed, careful examination of PNH PMN with bi-modal CD59 suggests that a population of cells has an intermediate CD59 phenotype (Fig. 1 *G*). To examine this question on a single-cell basis, we performed simultaneous evaluation of different GPI-anchored proteins ($Fc\gamma RIII_{PMN}$/DAF, $Fc\gamma RIII_{PMN}$/CD59, and DAF/CD59) by multi-color flow cytometry. All PMN expressing normal levels of $Fc\gamma RIII_{PMN}$ showed normal levels of DAF and CD59 (Fig. 4, *A* and *C*). Similarly, all DAF^+ PMN expressed normal levels of both DAF and FcγRIII (Fig. 4 *A*). Interestingly, while CD59 normal positive PMN expressed normal levels of FcγRIII and $CD59^-$ PMN were $Fc\gamma RIII_{PMN}$ intermediate, a distinct population of CD59 intermediate/$Fc\gamma RIII_{PMN}$ intermediate PMN was also observed (Fig. 4 *C*). However, no PMN were found to be DAF (or CD59) normal/$Fc\gamma RIII_{PMN}$ intermediate or DAF (or CD59) negative/$Fc\gamma RIII_{PMN}$ normal (Fig. 4, *A–C*). These results suggest an intrinsic rank order in the impairment of expression of GPI-anchored proteins in PNH cells.

We also examined the simultaneous expression of DAF and CD59 on the same PNH PMN (Fig. 4 *D*). Consistent with the results presented for DAF/$Fc\gamma RIII_{PMN}$ and CD59/$Fc\gamma RIII_{PMN}$, unaffected cells were observed (DAF normal/CD59 normal), and affected cells were either $DAF^-/CD59^-$ or DAF^-/CD59 intermediate. Thus, while absolute levels of expression of these different GPI-anchored molecules can be dissociated, no $DAF^+/CD59^-$ or DAF^-/CD59 normal cells could be demonstrated.

Analysis of NK cells confirmed that a generalized reduction of FcγRIII was not present on affected cells in PNH. Both DAF and CD59 were found to be expressed on NK cells (Fig. 5 *A* and results not shown). Two-color analysis of PNH lymphocytes demonstrated that both DAF^+ and DAF^- cells expressed normal and equal levels of $Fc\gamma RIII_{NK}$ (Fig. 5 *B*). The same was also true for both $CD59^+$ and $CD59^-$ cells (Fig. 5 *C*). Thus, the observation that both DAF and CD59 positive and negative NK cells expressed equal amounts of FcγRIII, coupled with PI-PLC and cDNA data (15–17, 20, 21, 45), confirms that $Fc\gamma RIII_{NK}$ has a single transmembrane linkage form.

Functional consequences of reduced FcγRIII on PMN. To investigate the functional consequences of the reduced level of expression of $Fc\gamma RIII_{PMN}$, we examined the phagocytosis of EA and E-Con A, which are FcγRII/FcγRIII- and FcγRIII-dependent probes, respectively (27). Analysis with MAb IV.3 demonstrated that the level of FcγRII on PNH PMN was indistinguishable from the normal control (mean fluorescence intensity: 135±4 and 131±5, respectively). Likewise, the absolute levels of %P for EA were indistinguishable from the normal control (Table I) and the absolute levels for the PI were either identical to or larger than the normal control (PNH patients with uniform intermediate $Fc\gamma RIII_{PMN}$ and bimodal $Fc\gamma RIII_{PMN}$, respectively). Phagocytosis of E-Con A was reduced as expected for an FcγRIII-specific probe (Table I). The amount of decrement in %P and PI was largest for PNH PMN with uniform intermediate FcγRIII ($P < 0.003$ and $P < 0.02$ for the PNH patients vs. normal controls). In addition, in the bimodal $Fc\gamma RIII_{PMN}$ PNH patients, the %P and PI for the E-ConA probe correlated with the degree of FcγRIII decrement ($r = 0.55$ and 0.61), with PMN having the lowest expression of $Fc\gamma RIII_{PMN}$ demonstrating the most profound decreases in %P and PI.

Discordant expression of CD14 and DAF on monocytes. To examine whether the discordant expression of GPI-anchored

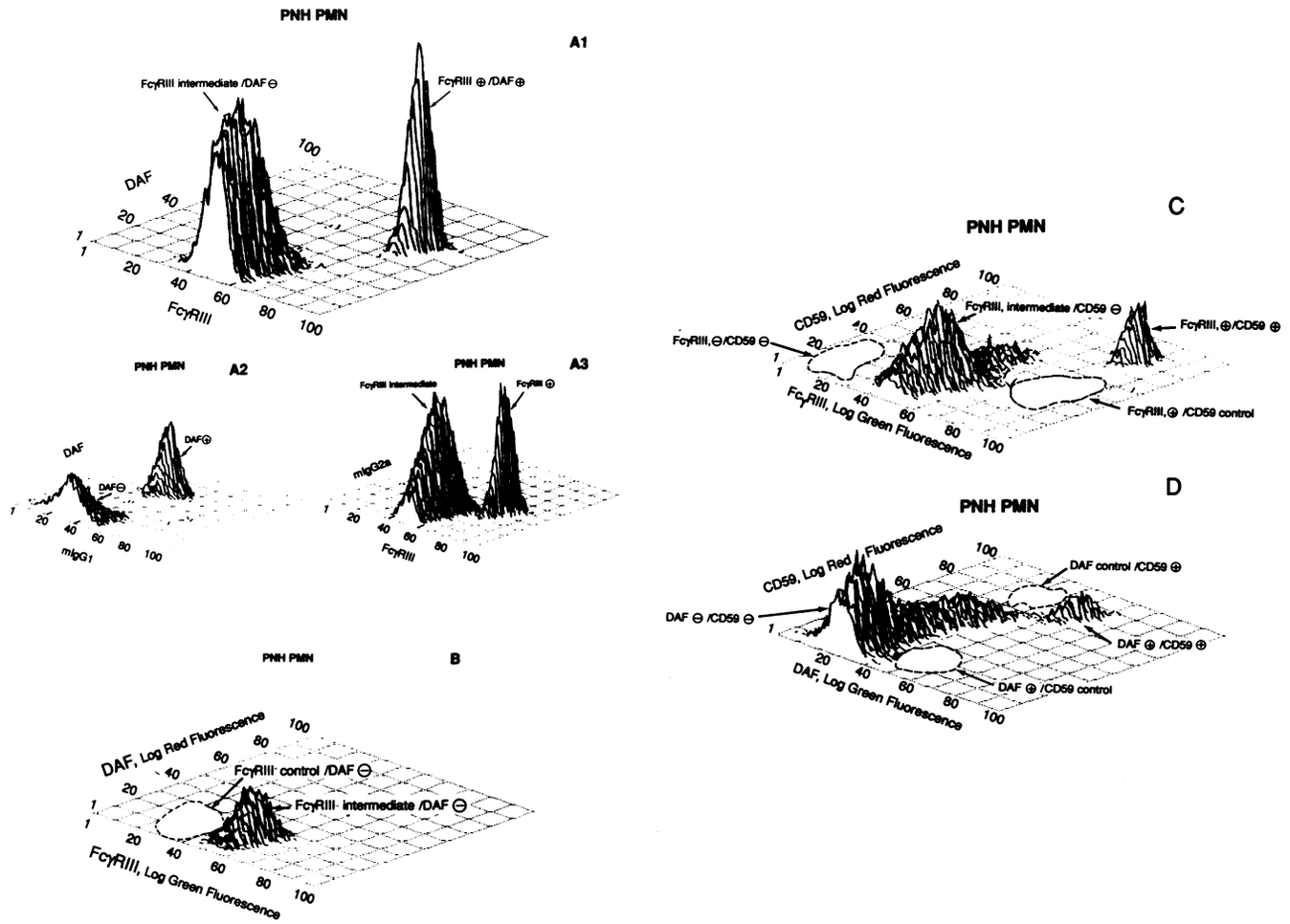

Figure 4. Discordant expression of FcγRIII and DAF/CD59 on PNH PMN. (*A*) Fresh PMN from PNH patient 1 show $DAF^-/Fc\gamma RIII$ intermediate cells and 15% normal cells ($DAF^+/Fc\gamma RIII^+$) (*A1*). PMN were analyzed by two-color flow cytometry for the expression of DAF and FcγRIII as described in the Methods. (*A2*) The anti-DAF/anti-FcγRIII mAb isotype control (anti-DAF/mIgG1). (*A3*) The anti-FcγRIII/anti-DAF isotype control (anti-FcγRIII/mIgG2a). The three and one half decade range of fluorescence intensity in this figure, and all in other two-color cytograms, is represented as 1–100 linear units (9 U = twofold difference in intensity). (*B*) PMN from PNH patient 5 show 100% $DAF^-/Fc\gamma RIII$ intermediate cells. The mIgG1/anti-DAF control is shown as a single contour line. The anti-FcγRIII/mIgG2a control was highly overlapping with the anti-FcγRIII/anti-DAF stained cells and is not shown for clarity. To examine whether the $DAF^-/Fc\gamma RIII$ intermediate phenotype resulted from the relative sensitivity of our detection systems, we analyzed both DAF and FcγRIII on PI-PLC–treated PMN from a normal donor. PI-PLC reduced the level of expression of both markers by ~ 75% as previously reported (3, 5, 6, 8, 15–20, 30). Treatment did not render DAF undetectable (mean fluorescence intensity, 62; MAb isotype control, 42) nor did it reproduce the PNH phenotype. (*C*) PMN from PNH patient 1 show $CD59^-/Fc\gamma RIII$ intermediate cells, CD59 intermediate/FcγRIII intermediate cells, and 12% $CD59^+/Fc\gamma RIII^+$ cells. Isotype controls, where appropriate, are shown as single contour lines. (*D*) PMN from PNH patient 1 show $CD59^-/DAF^-$ cells, CD59 intermediate/DAF^- cells, and 12% $CD59^+/DAF^+$ cells. Isotype controls, where appropriate, are shown as single contour lines.

markers on PMN from our PNH patients was characteristic of other leukocytes in the same patients, we investigated the expression of DAF, CD59, and CD14 (another GPI-linked protein [42, 43]) on monocytes. Blood monocytes, identified by light scattering and by characteristic MAb IV.3 immunofluorescence, demonstrated two patterns of DAF, CD59, and CD14 expression by single-color immunofluorescence. In the four patients with bimodal distributions of $Fc\gamma RIII_{PMN}$, DAF, and CD59 on their PMN, bimodal expression of DAF, CD59, and CD14 on monocytes was observed (Fig. 6, *A, D,* and *G*). All three markers were expressed at either normal levels (Fig. 6, *A* and *C, D,* and *F;* Fig. 2, *G* and *I*) or were undetectable. The proportion of normal cells was about the same for the monocytes and the PMN. The two patients with DAF^- and $CD59^-/Fc\gamma RIII$ intermediate PMN were uniformly negative for DAF, CD59, and CD14 expression on monocytes (Fig. 6, *B, E,* and *H*).

Finally, we examined blood monocytes by two-color flow cytometry for the simultaneous expression of DAF/CD14 and DAF/CD59. The monocytes of the two PNH patients with uniformly intermediate $Fc\gamma RIII_{PMN}$ expression were negative for both DAF and CD14 (Fig. 7 *B*). The other PNH patients with bimodal $Fc\gamma RIII_{PMN}$ usually demonstrated concordant expression of CD14/DAF and DAF/CD59 on their monocytes with either unaffected cells (DAF normal/CD59 normal/CD14 normal) or affected cells ($DAF^-/CD59^-/CD14^-$) (Fig. 7, *A* and *C*). A third population of cells (8.1–8.9% of the total monocytes, not observed in the four normal controls examined),

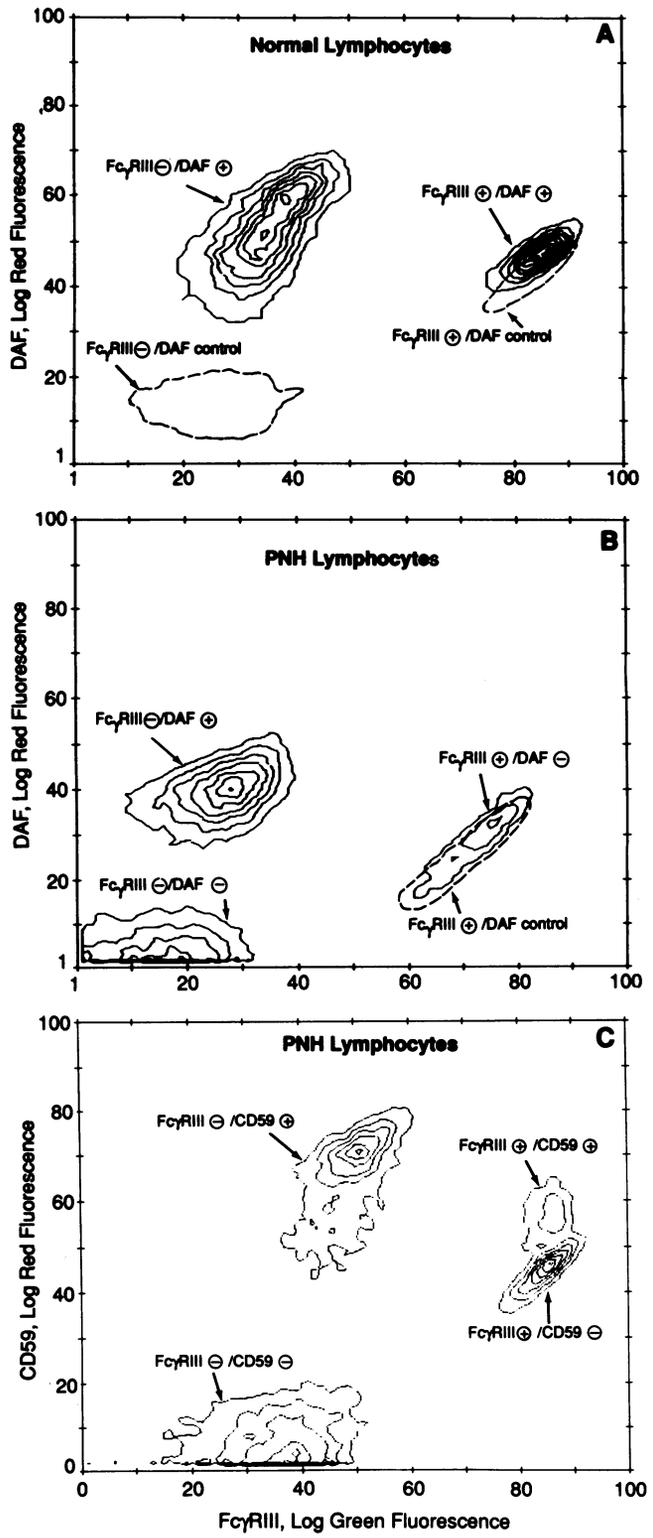

Figure 5. Dissociation of Expression of FcγRIII and DAF/CD59 on Lymphocytes (NK Cells). (*A*) Simultaneous expression of FcγRIII and DAF on NK cells from a normal donor. Cells were stained with anti-DAF MAb IA10 and anti-CD16 MAb 3G8-biotin + avidin-FITC and analyzed by flow cytometry with appropriate controls as described in the Methods. For clarity, important two-color isotype controls are shown as single dotted contour line outlining the cell populations. Other controls were superimposable with shown cell populations (see Methods). (*B*) PNH patient 2 shows both DAF^+ (10% of the $Fc\gamma RIII^+$ cells) and DAF^- NK cells ($Fc\gamma RIII^+$). This same patient was bimodal for leukocyte DAF. (*C*) PNH patient 3 shows both $CD59^+$ and $CD59^-$ NK cells ($Fc\gamma RIII^+$). This same patient was bimodal for leukocyte DAF.

Table I. Functional Consequences of Reduced $Fc\gamma RIII_{PMN}$ Expression

Donor	EA		E-ConA	
	%P	PI	%P	PI
Controls (n = 6)	60±12	100±30	59±14	91±34
PNH patients: uniform intermediate $Fc\gamma RIII_{PMN}$ (n = 2)	53±22	86±57	17±3*	20±4‡
PNH patients: bimodal $Fc\gamma RIII_{PMN}$ (n = 4)	75±22	200±87§	45±21	78±55

PMN from all six PNH patients and from healthy controls were incubated with EA or E-ConA. After lysis of noninternalized erythrocytes, phagocytosis by 500 PMN was quantitated by light microscopy. Results are expressed as percent phagocytosis (the percentage of PMN with one or more internalized targets) and phagocytic index (the number of internalized targets per 100 PMN).
* $P < 0.003$ for the PNH patients vs. normal controls.
‡ $P < 0.02$ for the PNH patients vs. normal controls.
§ $P < 0.05$ for the PNH patients vs. normal controls.

were DAF normal/$CD14^-$ (Fig. 7 *A*). To confirm that the cells in the monocyte light scatter gate were indeed monocytes, we examined these cells for the expression of T3 and B1 (T and B cell markers, respectively) and for the quantitative display of FcγRII with MAb IV.3. MAb IV.3, which recognizes the monocyte but not the lymphocyte isoform of FcγRII, uniformly stained both the $CD14^+$ and $CD14^-$ cells (Fig. 7 *D*) with characteristic intensity (38). Based on these multiple lines of evidence, both the $CD14^+$ and the $CD14^-$ cells were monocytes and not contaminating lymphocytes in the monocyte light scatter gate. Thus, discordant expression of GPI-anchored molecules in affected PNH cells is not restricted to FcγRIII on PMN.

Discussion

FcγRIII (CD16), expressed on polymorphonuclear and some mononuclear cells, is one of three families of human FcγRs. This receptor participates in the handling of model immune complexes in humans and subhuman primates (24–26). Because FcγRIII on PMN has a GPI anchor (15–21), and patients with PNH have a biochemical lesion in the glycolipid anchor pathway (2, 9–14), PNH PMN presented a potential clinical paradigm to assess the impact of $Fc\gamma RIII_{PMN}$ deficiency. Thus, we undertook a detailed study of the expression and function of FcγRIII on PMN in six PNH patients. Contrary to expectation, PNH patients showed relative preservation of CD16 expression and essentially normal handling of IgG-opsonized particles. $Fc\gamma RIII^-_{PMN}$ PMN were not observed in any of the PNH patients that we examined. Discordant expression of several different GPI-anchored markers in PNH PMN suggests that differences in anchor biochemistry or assembly must be important.

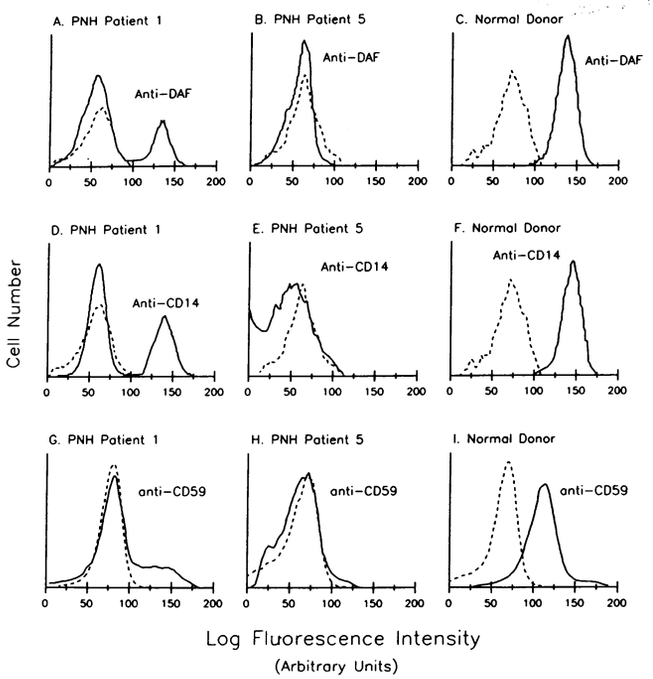

Figure 6. Expression of GPI-linked proteins on monocytes from PNH patients and normal controls. Fresh mononuclear cells from PNH patients 1 (*A, D, G*) and 5 (*B, E, H*) and from a healthy control (*C, F, I*) were incubated with anti-DAF MAb IA10 (*A–C*), anti-CD14 MAb LeuM3 (*D–F*), anti-CD59 MAb MEM-43 (*G–I*), or control mIgG1 or mIgG2a (– –), stained with PE-conjugated goat anti-mouse IgG, and analyzed by flow cytometry with gating on the monocyte population. The bimodal CD14, DAF, and CD59 (*A, D, G*) expression was characteristic of the four PNH patients with bimodal DAF/CD59/FcγRIII PMN, while the remaining two patients were $CD14^-$, DAF^-, and $CD59^-$ (*B, E, F*).

One molecular mechanism to explain preserved, but reduced, expression of $Fc\gamma RIII_{PMN}$ would be the existence of two membrane linkages, transmembrane and GPI anchor, as observed with LFA-3 or N-CAM (31). Analysis of mRNA from normal PMN has found evidence only for transcription of the gene for the PMN isoform (FcγRIII-1) that encodes for the GPI-anchored form (15). Translation and expression of the FcγRIII-2 gene in PNH affected PMN is unlikely since the predicted molecular weight of the protein derived from this gene (FcγRIII-2) is 6 kD larger than that normally expressed in PMN (20, 49) and the actual molecular weight of the receptor from the affected PNH PMN is indistinguishable from normal PMN (Fig. 3). Alternatively, the recent demonstration that co-transfection of the γ chain of FcεRI with FcγRIII-1 in GPI anchor–negative L cells facilitates the expression of a transmembrane, PI-PLC–resistant receptor (17, 30, 45) suggests another mechanism of $Fc\gamma RIII_{PMN}$ expression in PNH cells: expression of an unprocessed transmembrane form of the FcγRIII-1 gene product. This form of FcγRIII, expressed in L cells with γ chain, is 3 kD larger than normal $Fc\gamma RIII_{PMN}$ due to the presence of the uncleaved transmembrane domain (17, 30, 45). However, since the intermediate $Fc\gamma RIII_{PMN}$ from PNH cells was identical in molecular weight to normal control $Fc\gamma RIII_{PMN}$ and showed normal PI-PLC sensitivity, expression of such a transmembrane form of $Fc\gamma RIII_{PMN}$ in PNH is unlikely.

Another possible explanation for the negative/normal DAF (and CD59) and intermediate/normal FcγRIII distributions on PMN is the occurrence of several independent defects in GPI-anchor biosynthesis within a given patient. Thus, a patient might have normal cells, DAF^- or $CD59^-/Fc\gamma RIII_{PMN}$ normal cells and DAF or CD59 normal/$Fc\gamma RIII_{PMN}$ intermediate cells. Three lines of evidence argue against this model. First, for any given patient, the percentages of cells assigned to each cell population cannot be easily reconciled (Fig. 1). Second, this explanation cannot account for patients with uniformly intermediate $Fc\gamma RIII_{PMN}$ but negative DAF (Figs. 1 and 4). But most importantly, two-color flow cytometry shows that on a single-cell basis, GPI-linked markers are affected concomitantly, if not equally, in the same cells (Fig. 2). Thus, independent clonal lesions within a given patient, each selectively affecting only one protein species, appear highly unlikely, and a differential sensitivity of GPI-linked proteins to the PNH lesion(s) is a more tenable interpretation. The pathophysiological basis for this differential sensitivity to the lesion is unknown at present.

A formal possibility for the $DAF^-/Fc\gamma RIII_{PMN}$ intermediate phenotype is a "threshold for detection" artifact in our measurement systems. We believe that DAF negativity in the

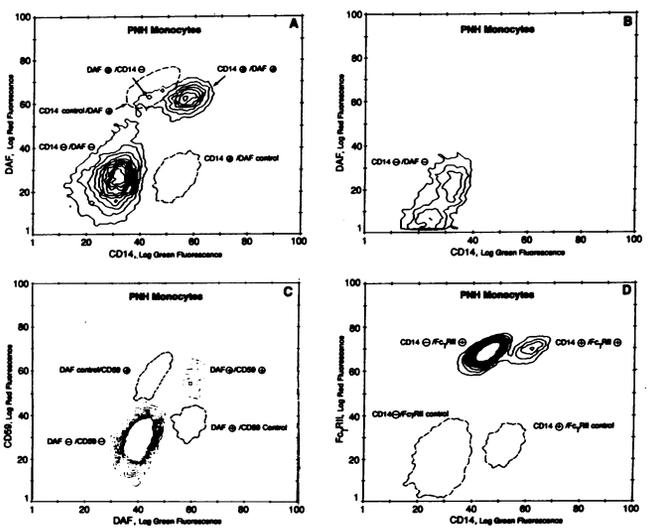

Figure 7. Concordant and discordant expression of DAF and CD14 on PNH monocytes. (*A*) Discordant expression of CD14 and DAF on monocytes from PNH patient 3. In addition to $DAF^+/CD14^+$ and $DAF^-/CD14^-$ cells, $DAF^+/CD14^-$ cells are observed. Fresh mononuclear cells from PNH patient 3 (with bimodal DAF/FcγRIII on PMN) were used. Cells were stained with anti-DAF MAb IA10 and anti-CD14 MAb MO2-FITC and analyzed by flow cytometry with appropriate controls as described in Fig. 3 (and see Methods). (*B*) Complete lack of CD14 and DAF expression on monocytes from PNH patient 5 (with DAF^-/FcγRIII intermediate PMN). Cells were stained as above. Both isotype controls were identical to the $CD14^-/DAF^-$ cell population, and for clarity are not shown. (*C*) Concordant expression of DAF and CD59 on monocytes from PNH patient 1 (with bimodal DAF/CD59/FcγRIII PMN). Cells were stained with anti-DAF mAb IA10-FITC and anti-CD59 mAb MEM-43 and analyzed as described above. (*D*) $CD14^-$ cells in the monocyte light scatter gate are $Fc\gamma RII^+$. Cells from PNH patient 3 were stained and analyzed as above except anti-FcγRII MAb IV.3 was used instead of anti-DAF. All cells were found to be $Fc\gamma RII^+$, confirming that the $CD14^-$ cells were monocytes, not contaminating lymphocytes (which are $Fc\gamma RII^-$ or $Fc\gamma RII^{dim}$ with MAb IV.3).

context of $Fc\gamma RIII_{PMN}$ intermediacy is a true phenotype since the ability of the anti-DAF antibody to detect reduced levels of DAF with comparable sensitivity to anti-CD16 was addressed in several independent ways. First, for the two-color analysis, baseline DAF immunofluorescence was equal in intensity to $Fc\gamma RIII_{PMN}$ immunofluorescence through the use of a PE-conjugated second reagent for DAF and a FITC-conjugated reagent for $Fc\gamma RIII_{PMN}$. Second, after PI-PLC treatment of normal PMN, which led to a > 75% decrease in surface DAF, DAF immunofluorescence remained well above the isotype control (legend to Fig. 4 *B*). Third, analysis of NK cells (defined by CD16 immunofluorescence), which express very low levels of DAF (21, 50), clearly demonstrated DAF immunofluorescence. Interestingly, $CD16^-$ (but $CD56^+$) NK cells have been identified in peripheral blood and represent ~ 10% of the total NK cells (and < 1% of the total lymphocyte population) (51). The level of DAF expressed on these cells and their presence in PNH await further analysis. Finally, the finding of discordant expression of CD59 (which has a higher baseline level of expression than DAF) and $Fc\gamma RIII_{PMN}$ makes it most likely that both the $DAF^-/Fc\gamma RIII_{PMN}$ intermediate and the $CD59^-/Fc\gamma RIII_{PMN}$ intermediate phenotypes represent true differences in GPI-anchored protein expression. Indeed, there are suggestions that erythrocytes may also reflect discordant expression of GPI-anchored molecules such as DAF and homologous restriction factor (52, 53), DAF and acetylcholinesterase (54), and DAF and LFA-3 (54). However, $Fc\gamma RIII_{PMN}$ is unique among these GPI-linked proteins in that expression is preserved in all affected cells; $Fc\gamma RIII^-_{PMN}$ PMN were never observed.

Despite intermediate expression of $Fc\gamma RIII_{PMN}$, PMN retained a significant ability to perform Fcγ receptor–mediated phagocytosis (Table I). With IgG-opsonized erythrocytes, phagocytic ability remained near the normal range. This observation probably reflects the efficient collaboration of FcγRII and $Fc\gamma RIII_{PMN}$ in handling this probe. In contrast, E-ConA, which ligate $Fc\gamma RIII_{PMN}$ but not FcγRII (27, 37), were handled less efficiently by PNH PMN, with the absolute levels of PI and %P being lower than the normal range. The magnitude of this decreased phagocytic capacity also was in direct relationship to the level of $Fc\gamma RIII_{PMN}$ expression in the sample. Perhaps more important, however, is that in the absence of complete FcγRIII deficiency, PMN phagocytic function for classically opsonized probes was preserved and for nonclassical lectin-like probes was reduced but not negative. These functional observations, coupled with normal FcγRIII expression on NK cells (Fig. 5) (21) and cultured monocytes (22, 23), suggest that the clinical impact of the PNH lesion(s) on Fcγ receptor-mediated processes would be minimal.

The fundamental molecular mechanism(s) of defective GPI-linked protein expression in affected cells in PNH is unclear. Normal DAF mRNA transcripts have been detected in affected PMN (55). These mRNA are translated (in affected, surface DAF^- cells) to yield an abnormally sized nascent pro-DAF polypeptide that is processed in the Golgi complex (56). In addition, smaller forms of high molecular weight DAF have been found on DAF^+ PNH erythrocytes relative to normal erythrocytes (57). Discordant expression of GPI-anchored proteins on leukocytes and diminished expression of LFA-3 and acetylcholinesterase on DAF^- erythrocytes (54) indicate heterogeneity in the expression of GPI-anchored proteins in PNH. Whatever the lesion(s) in PNH cells, it must provide a mechanism of differential sensitivity of GPI-linked protein expression by both leukocytes and erythrocytes. This selective preservation of certain molecules, like $Fc\gamma RIII_{PMN}$ (CD16), provides for a rich diversity in the clinical expression of disease manifestations in PNH. Further studies on the biochemistry of the expression of the GPI linked $Fc\gamma RIII_{PMN}$ and on the biosynthetic mechanisms of GPI linkages will be needed to understand the mechanism of expression of $Fc\gamma RIII_{PMN}$ on affected cells in PNH.

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