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The major Fc receptor in blood has a phosphatidylinositol anchor and is deficient in paroxysmal nocturnal haemoglobinuria

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Fc receptors on phagocytic cells in the blood mediate binding and clearance of immune complexes, phagocytosis of antibody-opsonized microorganisms, and potently trigger effector functions, including superoxide anion production and antibody-dependent cellular cytotoxicity. The Fc receptor type III (FcγR III, CD16), present in 135,000 sites per cell 1 on neutrophils and accounting for most of FcR in blood, unexpectedly has a phosphatidylinositol glycan (PIG) membrane anchor. Deficiency of FcγR III is observed in paroxysmal nocturnal haemoglobinuria (PNH), an acquired abnormality of haematopoietic cells[2] affecting PIG tail biosynthesis or attachment[3], and is probably responsible for circulating immune complexes[4] and susceptibility to bacterial infections associated with this disease[5]. Although a growing number of eukaryotic cell-surface proteins with PIG-tails are being described[6,7], none has thus far been implicated in receptor-mediated endocytosis or in triggering of cell-mediated killing. Our findings on the FcγR III raise the question of how a PIG-tailed protein important in immune complex clearance *in vivo*[8,9] and in antibody-dependent killing[10] mediates ligand internalization and cytotoxicity. Together with our results, previous functional studies on FcγR III and FcγR II[11,12] suggest that these two receptors may cooperate and that the type of membrane anchor is an important mechanism whereby the functional capacity of surface receptors can be regulated.

Three different types of FcγR have been distinguished in humans using monoclonal antibodies[13] (mAb). FcγR III (CD16) of relative molecular mass (M_r) 50-70,000 (50-70K) is found on neutrophils, large granular lymphocytes, and macrophages, but not on monocytes. FcγR III was first identified with a mAb (3G8) that blocked immune complex binding to neutrophils[1] and subsequently with other mAb of the CD16 cluster[14]. FcγR II (CDw32) is a 40K receptor on neutrophils, monocytes, eosinophils, platelets and B cells[13]. FcγR I is a 72K protein and is found on monocytes[13]. FcγR III and FcγR II have low affinity for monomeric IgG and thus preferentially bind immune complexes by multiple receptor-ligand interactions, whereas FcγR I is sufficiently high affinity to bind monomeric IgG.

Our first evidence that FcγR III is anchored by PIG came from studies on leukocytes from patients with paroxysmal nocturnal haemoglobinuria (PNH). PNH is an acquired defect of haematopoietic precursor cells in either the biosynthesis or the attachment of the PIG tail and may affect clonal progeny in the erythroid, monocytic, granulocytic, and thrombocytic lineages[2,15,16]. Previous studies on erythrocytes and leukocytes from PNH patients have demonstrated a selective deficiency of PIG-tailed proteins, including decay accelerating factor (DAF), acetylcholinesterase, alkaline phosphatase and the PIG-anchored form of lymphocyte function-associated antigen 3 (LFA-3), (refs 3, 6 and 7). The deficiency of DAF accounts for susceptibility of erythrocytes to complement-mediated lysis in PNH. However none of these previously identified deficiencies can explain the occurrence of circulating immune complexes[4] and the 20% and 50% of mortalities caused by bacterial infections and thrombosis respectively[5].

Quantitation of FcγR III expression using immunofluorescence flow cytometry show that it is markedly deficient on PNH neutrophils (Fig. 1*a*). This deficiency was found in all six patients studied (D.E., S.B., J.M., J.E., B.I., C.G.) and results with five different CD16 (FcγR III) mAb were identical. Some patients such as J.E. (Fig. 1*a*, curve 3) showed normal as well as deficient granulocyte clones. Patients showed consistent variation in the extent of deficiency in the abnormal clone. The amount of FcγR III expression on affected cells ranged from 2% (patient D.E.) to 19% (patient J.E.) averaging 7% of normal, perhaps reflecting the degree of penetrance of the acquired defect in PNH. In all cases, deficiency of FcγRIII paralleled deficiency of DAF. In contrast, deficient neutrophils expressed normal levels of HLA-A,B, LFA-1, Mac-1 and FcγR II (CDw32) (Fig. 1*a*). PNH monocytes showed normal expression of FcγR I and II, although they were deficient in DAF (Fig. 1*b*). These results suggested that the neutrophil FcγR III has a PIG tail, whereas the FcγR I and FcγR II do not.

PIG-anchored proteins can be specifically cleaved from cell surfaces with phosphatidylinositol-specific phospholipase C[3,6,7] (PIPLC). We therefore investigated the susceptibility of Fc receptors to PIPLC (Table 1). PIPLC released 75-84% of the cell surface FcγR III and DAF from healthy granulocytes, while FcγR II, HLA-A,B and LFA-1 were unaffected. On monocytes, PIPLC released 84% of DAF whereas it had no effect on FcγR II, FcγR I, HLA-A,B and LFA-1. Results with PIPLC prepared from *S. aureus* and *B. thuringiensis* were identical and show that FcγR III on neutrophils, but not FcγR II on the same cells or FcγR I and II on monocytes, have PIG anchors. Lack of a PIG anchor on FcγR II is consistent with the prediction (from cDNA sequence[17]) that it possesses a transmembrane domain and a 76 residue hydrophilic cytoplasmic tail.

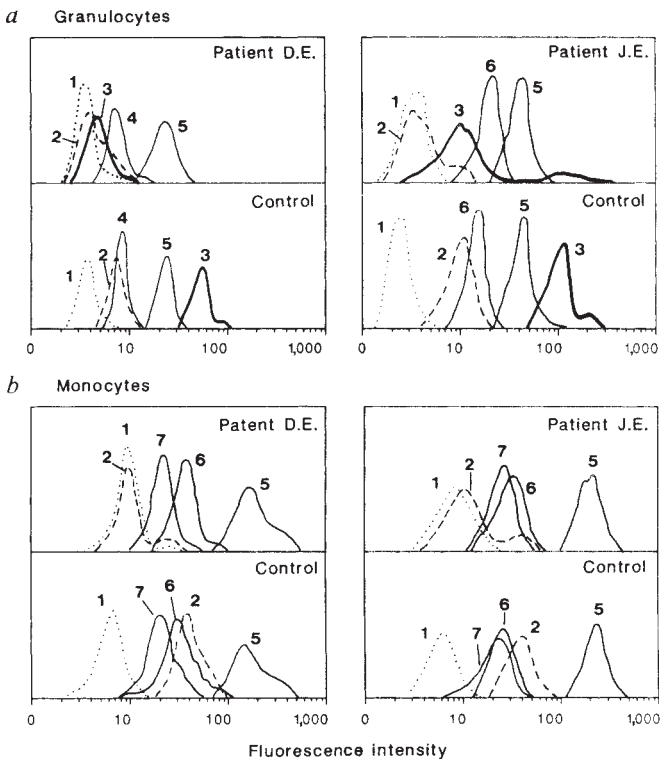

Fig. 1 Immunofluorescence flow cytometry analysis of granulocytes (*a*) and monocytes (*b*) from PNH patients and normal controls. PNH patient D.E. had type III erythrocytes and patient J.E. had type III and type I erythrocytes. 1, ... X63 control; 2, - - - - DAF; 3, —— FcγR III (3G8); 4, LFA-1; 5, HLA-A, B; 6, FcγR II; 7, FcγR I (4-7, ——).

Methods. Blood samples were collected from PNH patients and from healthy individuals in acid citrate dextrose (ACD) as anticoagulant. Leukocyte-rich plasma was separated from erythrocytes by 6% dextran T-500 (Pharmacia) sedimentation, overlaid on Histopaque-1077 (Sigma Chemical Co.) and centrifuged at 1,000 *g* for 25 min. Mononuclear cells were recovered from the interface and granulocytes were recovered from the pellet with some erythrocytes. The contaminating erythrocytes were removed by hypotonic lysis with water for 20 s. For some experiments granulocytes were pretreated with fMet-Leu-Phe as described[26]. Cells were washed five times, stained with hybridoma culture supernatants diluted two fold or ascites diluted 400-fold with fetal calf serum (FCS)/RPMI 1640 followed by FITC conjugated goat anti-mouse IgG or IgM (Zymed), and subjected to flow cytometry as previously described[26]. Monoclonal antibodies have been previously referenced: 1A10 to DAF (ref. 16), 3G8 to FcγR III (ref. 1); W6/32 to HLA-A,B, TS1/22 to LFA-1, LM2/1 to Mac-1 (ref. 26), and 32 to FcγR I (ref. 27) or were from the third international workshop on human leukocyte differentiation antigens[14]: 2E1 and CIKM5 to FcγR II, and CLBFc gran-1, MG38, G2G4, BW243/41 and G7E11 to FcγR III. P3X63 myeloma IgG and OCH.217 CD2 IgM antibody were used as non-binding controls for IgG and IgM staining respectively.

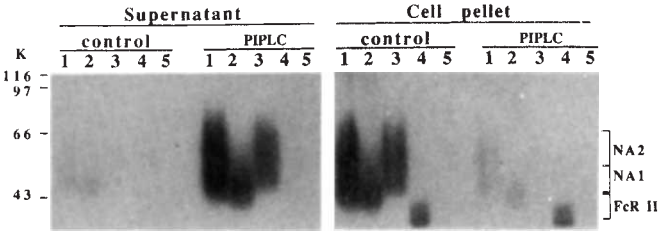

Fig. 2 SDS-PAGE of FcγR III after PIPLC treatment. FcγR was precipitated from the supernatant or cell lysate after treatment of ^{125}I-labelled neutrophils, with or without PIPLC. Lane 1, FcγR III precipitated by CLBFc gran-1; lane 2, NA1 allotype of FcγR III precipitated by MG38; lane 3, NA2 allotype of FcγR III precipitated by CLBFc gran-1 after preclearing with MG38; lane 4, FcγR II precipitated by CIKM5; lane 5, X63 control precipitate, Size markers are indicated to the left.

Method. Granulocytes were surface iodinated with ^{125}I using 1,3,4,6-tetrachloro-3α,6α-diphenyl glycoluril[28]. Aliquots of the iodinated cells were washed three times with HBSS/HEPES, *p*H 7.4, and treated with *Bacillus thuringiensis* PIPLC as described under Table 1. Cells were centrifuged at 340 *g*. The resultant supernatant was further centrifuged at 100,000 *g* and saved. The cell pellet was washed with 10% FCS/RPMI and lysed in 1% Triton X-100 in 50 mM Tris/HCl buffer, *p*H 8.0, containing 0.02% azide, 1% bovine haemoglobin, 5 mM iodoacetamide, 1 mM PMSF, 1 mM diisopropyl fluorophosphate and 1% aprotinin. The lysate and 100,000 *g* supernatant were dialysed overnight against 10 mM Tris, 145 mM NaCl and 0.02% azide. Samples (20 μl) were incubated at 4 °C and 18 μl ascites (diluted 1:50), 5 μl rabbit anti-mouse IgG (Zymed) and 30 μl protein A-agarose (Genzyme) were added sequentially at 4 h intervals. The resulting immunoprecipitates were washed three times with lysis buffer (diluted 1:10), once with 50 mM Tris/HCl, *p*H 8.0 and analysed by SDS PAGE (10% gels) under non-reducing conditions The gel was dried and autoradiographed; only the relevant portion is shown.

FcγR III is polymorphic[18]. The alloantigens NA1 and NA2 are distinguished by human alloantisera, by size in SDS-PAGE, and by certain CD16 mAb. The results in Table 1 were obtained with NA1, NA1 homozygotes and NA1, NA2 heterozygotes. The amount of release with PIPLC was similar whether measured with MG38 mAb specific for NA1 or with four other CD16 mAb (CLB FcR gran-1, BW243/4, G2G4, and G7E11) which react with both alloantigens (Table 1). These CD16 MAb have been placed in three distinct groups by Tetteroo *et al.*[14], based on intensity of staining or differential reactivity with neutrophils and large granular lymphocytes.

FcγR III bearing NA1 has higher mobility than that of NA2 (refs 14, 18). Sequential immunoprecipitation using a NA1-specific allotypic mAb and a monotypic mAb showed that both NA1 and NA2 allotypes were released by PIPLC treatment (Fig. 2 lanes 2 and 3). FcγR II (Fig. 2 lane 4) and LFA-1 and Mac-1 (data not shown) were not released. A small amount of FcγR III was released in the absence of PIPLC, possibly due to the action of endogenous phospholipases. The amount of spontaneous release appeared to be higher with neutrophils which had been prestimulated with fMet-Leu-Phe (not shown). Soluble FcγR III has been detected in plasma[19].

We conclude that the neutrophil FcγR III has a PIG anchor. FcγR III on neutrophils accounts for ~50% of all Fcγ receptors in the blood and 80% of those found on phagocytic cells (Table 2). Its deficiency is likely to underlie the presence of circulating immune complexes in PNH[4] and to be related to the susceptibility of PNH patients to bacterial infections[5]. Administration of mAb or Fab to the FcγR III profoundly inhibits immune complex clearance *in vivo*[8,9], further supporting its important role. In addition to that on neutrophils, FcγR III in spleen red pulp and liver Kuppfer cells may be important in *in vivo* clearance[9]. The nature of the anchor on these cells remains to be determined.

The neural cell adhesion molecule (NCAM) and LFA-3 exist in both PIG- and polypeptide-anchored forms[7]. Although we cannot rule out a polypeptide-anchored form of FcγR III on neutrophils, 98% deficiency in PNH patients D.E. (Fig. 1) and J.M. (data not shown) suggest that if present at all, it would constitute ≤2% of the total receptor. Incomplete release of PIG-tailed proteins with PIPLC is always observed, most probably due to acylation at the 2-OH of inositol[7], and does not indicate the existence of a polypeptide-anchored form.

Table 1 Effect of PIPLC treatment on binding of FcγR mAbs to granulocytes and monocytes

	Fluorescence Intensity			
	Control	+PIPLC *S. aureus*	+PIPLC *B. thuringiensis*	% decrease (Mean ± s.d.)
Granulocytes				
DAF	2.1	0.7	0.3 ± 0.2	75 ± 9
FcγR III				
3G8	13.5	3.3	2.4 ± 0.7	78 ± 2
CLB FcR gran1	14.3	4.0	2.7 ± 0.9	77 ± 4
MG38	12.7	3.7	2.8 ± 0.9	75 ± 4
G2G4	22.6	—	3.2 ± 2.7	84 ± 14
G7E11	27.5	—	4.7 ± 3.0	83 ± 12
BW243/41	0.9	—	0.2 ± 0.12	76 ± 13
FcγR II	4.2	4.3	4.9 ± 0.26	0
LFA-1	2.1	2.3	2.3 ± 0.1	0
Mac-1	8.9	9.4	8.9 ± 0.1	0
HLA-A, B	10.7	11.6	11.4 ± 0.7	0
Monocytes				
DAF	10.6	—	1.7	84
FcγR I	5.4	—	6.0	0
FcγR II	7.8	—	8.5	0
HLA-A, B	62.8	—	68.1	0

Cells ($5\text{-}10 \times 10^6$ per ml) were resuspended in HBSS/HEPES *p*H 7.4 containing 1 mg ml^{-1} ovalbumin; treated with *S. aureus* (10 μg ml^{-1}) or *B. thuringiensis* (0.6 mmol min^{-1} ml^{-1}) PIPLC for 45 min at 37 °C and analysed by flow cytometry as described in Fig. 1. The antibody staining on control cells which were treated in the same way but without PIPLC was taken as 100%. For comparative purposes the control values were normalized. Monocytes and granulocytes were from different donors and were analysed at different times. Among FcγR III mAbs MG38 reacts only with NA1, whereas others react with both NA1 and NA2. The averaged results with *B. thuringiensis* PIPLC were from two homozygous (NA1, NA1) and a heterozygous (NA1, NA2) donor. The specific activity of *B. thuringiensis* PIPLC was 100-fold higher than *S. aureus* PIPLC (M. Low, personal communication).

An important question is whether the PIG anchor, with its diacylglycerol moiety present in only the outer leaflet of the membrane bilayer, can deliver signals. Although mAb-induced cross-linking of Thy-1 and Ly-6 on T cells can trigger functions such as mitogenesis[20,21], the natural ligands of these PIG-tailed proteins are unknown, and it is not clear whether Thy-1 or Ly-6 trigger physiologically. Because Fc receptors are potent physiological triggers of neutrophil and monocyte function, it is of considerable interest that the most abundant of these is PIG-tailed. Monoclonal antibodies and Fab fragments to FcRγ III have previously been found to block binding and hence internalization of soluble immune complexes and antibody-opsonized erythrocytes[1] to neutrophils, and to inhibit antibody-dependent killing of target cells[10]. This is not direct evidence for triggering by FcγR III, however, as both FcγR III and FcγR II will be engaged by immune complexes and thus may act synergistically. The roles of FcγR III and FcγR II in triggering have been partly resolved by antibody-redirected killing. Resting or IFN-γ-stimulated neutrophils kill chicken erythrocytes in the presence of anti-FcγR III Fab conjugated to anti-chicken erythrocyte Fab[11], indicating that a PIG-tailed receptor can trigger. But FcγR I induced by IFN-γ on neutrophils is a better trigger, despite its lower density. Assays on hybridoma cells bearing anti-FcR surface Ig show that FcγR III does not trigger neutrophil killing of tumour cells, whereas FcγR II present at a four-fold lower density does[12]. Studies on neutrophil superoxide anion generation suggest that FcγR III is required for binding of immune complexes and FcγR II has an important role in triggering[1,22].

Table 2 Fc receptors in blood

		Sites per cell* $\times 10^{-3}$	Blood concentration (nM)†
Neutrophil	FcγR II	31	0.22
	FcγR III	135	1.0
Monocyte	FcγR I	20	0.01
	FcγR II	36	0.014
B cell	FcγR II	38	0.02
Platelet	FcγR II	1.4	0.7

* Sites per cell from refs 1, 13, 23 and 24 and our own unpublished results.

† Calculated from cell concentrations in blood[25].

Thus functional studies together with our results suggest that a physiologically relevant PIG-anchored receptor, FcγR III, can trigger at least one type of neutrophil function (killing of erythrocytes[11]) but that it triggers less efficiently than the protein-anchored FcγR I and FcγR II (refs 11, 12). Although we cannot rule out influences of the extracellular domains of these receptors, the findings suggest that the type of membrane anchor may be an important mechanism for regulating the functional capacity of surface receptors. The FcγR III is present in more copies per cell (135,000) than any other known receptor on a circulating cell. Immune complexes must be removed from the circulation without causing disseminated neutrophil activation; otherwise severe immunopathological reactions ensue. The PIG anchor may allow efficient ligand binding and internalization to be coupled with relatively weak signalling.

The FcγR III is coexpressed on neutrophils with the FcγR II. The ability of mAb to either the FcγR II or FcγR III to inhibit neutrophil functions[1,10,22] suggests that these low affinity receptors may synergize in interactions with multivalent immune complexes. The high density of the FcγR III and its predicted fast mobility in the membrane bilayer[6,7] may suit it for capture of immune complexes, while the FcγR II may have the major role in triggering.

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