On the Interaction of IgG Subclasses with the Low Affinity FcγRIIa (CD32) on Human Monocytes, Neutrophils, and Platelets

Analysis of a Functional Polymorphism to Human IgG2

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Abstract

An allotypic form of the low affinity IgG Fc receptor FcγRIIa (CD32), termed low responder (LR) because of its weak reactivity with mouse (m) IgG1, interacts efficiently with human (h) IgG2. FcγRIIa^{LR} is the first known human FcR that binds this IgG subclass. In this study, we analyzed the role of FcγRIIa in binding of stable hIgG-subclass dimers, and in induction of T cell mitogenesis using chimeric anti-CD3 mAb. We demonstrate that the functional polymorphism to hIgG2 is expressed on the majority of FcγR-bearing peripheral blood cells: monocytes, neutrophils, and platelets. We were able to assess FcγRII-mediated IgG-binding without interference of other FcγR-classes, by blockade of FcγRI on monocytes, and by using neutrophils of an individual deficient for the FcγRIIIB gene. This study indicates as subclass specificity: hIgG3 > hIgG1,hIgG2 ≫ hIgG4 for FcγRIIa^{LR} and hIgG3,hIgG1 ≫ hIgG2 > hIgG4 for FcγRIIa^{HR}. Comparing the serum hIgG levels of individuals homozygous for the two FcγRIIa allotypic forms, we observed significantly lower hIgG2 serum levels in individuals expressing the hIgG2-binding LR allotypic form. This observation may implicate that FcγRIIa regulates hIgG subclass production or turnover in man. (*J. Clin. Invest.* 1992. 90:1537–1546.) Key words: chimeric anti-CD3 • T cell activation • serum IgG • CD16 • CD64

Introduction

Receptors specific for the Fc moiety of IgG (FcγR), expressed on a broad range of hematopoietic cells, create an important linkage between humoral and cellular defence mechanisms. On human leukocytes, three distinct classes of IgG Fc receptors (FcγR) are currently recognized. FcγRI (CD64) is a 72-kD high affinity receptor (Ka ~ $10^8 - 10^9$ M^{-1}) that is expressed constitutively on monocytes and macrophages, and is IFN-γ- and G-CSF-inducible on neutrophils (1, 2). FcγRII (CD32) and FcγRIII (CD16) display lower affinities (Ka < 10^7 M^{-1}) and primarily bind immune complexes. Both FcγRII and FcγRIII have been demonstrated on neutrophils, eosinophils, and macrophages. FcγRII is, furthermore, expressed on monocytes, B cells, basophils, and platelets (3, 4).

The subclass specificity of FcγRII (on platelets) was previously determined as human (h)IgG1 = hIgG3 > hIgG2,hIgG4 (5). This view may be incomplete; however, since cDNA cloning of FcγRII revealed multiple FcγRII-isoforms and allotypes (6–8), which may express differences in IgG subclass specificity. Three distinct genes termed hFcγRIIA, B, and C have been described, which encode highly homologous proteins regarding the extracellular parts, but which display strong structural divergence in cytoplasmic domains (6, 7, 9, 10). This indicates that differences in subclass specificity between the various forms, and differential expression on distinct cells may have major consequences for antibody effector functions. FcγRII is known to be polymorphic with respect to its interaction with mouse (m)IgG1 complexes (4, 11). It was recently shown that this functional polymorphism resides in a single Arg-His amino acid change at position 131 in FcγRIIa (12–14). Excitingly, it was found that this change also has implications for the interaction with hIgG: transfected fibroblasts expressing FcγRIIa (131-His), which was termed low responder (FcγRIIa^{LR}) because of its weak reactivity with mIgG1, appeared to bind hIgG2 dimers efficiently, in contrast to the FcγRIIa high responder (FcγRIIa^{HR}) allotypic form (13).

Recently, we developed a matched set of mouse/human chimeric anti-CD3 mAb (15). mAb directed against the multimolecular TcR/CD3 complex on the cell surface of human T cells, are capable of inducing T cell activation, both in vitro and in vivo (16). Accessory cells such as monocytes, however, are required to induce full activation and proliferation of the T cells and provide crosslinking of the anti-CD3 mAb via Fc receptors, as well as production of lymphokines such as IL-1 (11, 17). Using these antibodies, we demonstrated that this FcγRII polymorphism is functionally expressed in anti-CD3 mAb induced T cell activation, both with monocytes and FcγRIIa^{HR}- or FcγRIIa^{LR}-transfected fibroblasts as accessory cells (18).

A complication in linking the above observations to other cells expressing FcγRII is the potential coexpression of multiple isoforms of FcγRII, such as FcγRIIb, which expresses a distinct subclass specificity (Warmerdam, P. A. M., I. E. Oudÿk, P. W. H. I. Parren, N. A. C. Westerdaal, J. G. J. van de

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1. *Abbreviations used in this paper:* FcγR, IgG Fc receptor; GPI, glycosyl-phosphatidylinositol; GPI-PLC, GPI-specific phospholipase C; h, human; HR, high responder (to mIgG1); IMDM, Iscove's modified Dulbecco's medium; LR, low responder (to mIgG1); m, mouse; PLC, phospholipase C; PNH, paroxysmal nocturnal hemoglobinuria; TcR, T cell receptor.

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Winkel, and P. J. A. Capel. Submitted for publication), and coexpression of members of different FcγR classes on most blood cells (4). Furthermore, studies with hybrid antibodies have suggested that one IgG molecule may simultaneously interact with two distinct FcγR molecules (19), this might imply that potential cooperation between different classes of FcγR molecules may affect IgG binding avidity.

We now demonstrate the functional FcγRIIa polymorphism for hIgG2 complexes on the majority of FcγR bearing peripheral blood cells: monocytes, neutrophils, and platelets. Comparing IgG serum levels between groups of individuals homozygous for either FcγRIIa allotype, we found significantly lower serum hIgG2 levels in FcγRIIa^{LR} individuals. This observation may implicate that FcγRIIa^{LR}, which is the first FcR known to bind hIgG2 efficiently, may be involved in modulation of hIgG serum levels.

Methods

Monoclonal antibodies. Hybridoma cell line CLB-T3/4.1, producing a mIgG1,κ mAb reactive with the ε chain of the TcR/CD3 complex (20) and its heavy chain isotype variants have been described in detail (15, 21).

Anti-FcγRI mAb 197 (γ2a,κ) and mAb 22 (γ1,κ; FITC-conjugated), anti-FcγRII mAb IV.3 (γ2b,κ) and anti-FcγRIII mAb 3G8 (γ1,κ) were kindly provided by Medarex, Inc. (W. Lebanon, NH). Anti-FcγRII mAb 41H16 (γ2a,κ) was provided by Dr T. Zipf (University of Texas, Houston, TX), and anti-FcγRIII mAb CLB Gran1 (γ2a,κ) was from the CLB. Anti-CD28 mAb CLB-CD28/1 (γ1,κ) was provided by Dr R. van Lier (CLB, Amsterdam).

Purification of PBC. T cells were isolated by sheep E-rosette sedimentation from PBMC, obtained by Percoll density centrifugation from buffy coats, as described in (15). Residual monocytes were removed by plastic adherence. T cell purity was checked by measuring LPS-induced monocytic IL-6 production (22), which was found < 5 pg/ml IL-6 in an overnight incubation of 200,000 cells/ml, whereas unseparated PBMC produced 2,000 to 10,000 pg/ml.

Monocytes were isolated from PBMC by counterflow centrifugation, as described elsewhere (23), and were > 95% pure, as determined in cytocentrifuge preparations after staining for nonspecific esterase, and May Grünwald-Giemsa.

Granulocytes were separated from PBMC and platelets by centrifugation of trisodium citrate (14 mM) anticoagulated blood on 1.076 g/ml Percoll. Erythrocytes were removed from the granulocyte pellet by lysis in isotonic NH_4Cl on ice, resulting in a PMN preparation containing > 95% neutrophils (24). Induction of PMN-FcγRI was analyzed after incubation of cells in culture medium (see below) supplemented with 300 U/ml IFN-γ (Genentech, San Francisco, CA), inhibition analyses of IFN-γ induced effects were performed by supplementing inhibitory anti-IFN-γ mAb MD-1 (10 μg/ml) (25).

Platelet-rich plasma was obtained by centrifugation (15 min, 250 *g*) of EDTA (5 mM) anticoagulated blood. Platelets were then concentrated by centrifugation (7 min, 1,200 *g*) and three washes were performed with PBS/5 mM EDTA/0.5% FCS (26).

PMN expressing reduced levels of FcγRIII were isolated from two patients with diagnosed paroxysmal nocturnal hemoglobinuria (PNH) (27, 28). The neutrophil preparations from these patients contained ~ 80% and ~ 100% of (GPI-linked) FcγRIIIb deficient cells, respectively, and expressed ~ 10% of normal FcγRIIIb levels. FcγRIII-deficient (FcγRIII^{neg}) PMN were isolated from an FcγRIIIB-deficient donor. The absence of the FcγRIIIB gene, FcγRIIIb mRNA, and soluble FcγRIIIb in serum was previously demonstrated in a study by Huizinga et al. (29). PMN from this FcγRIIIb^{neg} donor showed a complete absence of FcγRIII expression in immunofluorescence studies (see Results; Fig. 4 *B*).

GPI-PLC treatment. FcγRIIIb was removed from normal PMN by incubation with GPI-specific phospholipase C (GPI-PLC). PMN were washed and incubated for 1 h at 37°C in Iscove's modified Dulbecco's medium (IMDM) supplemented with a 1:1,000 dilution of GPI-PLC. GPI-PLC isolated from *Bacillus thuringiensis* was kindly provided by Dr. T. Schumacher (Netherlands Cancer Institute, Amsterdam).

T cell proliferation assay. Highly purified T cells were stimulated with graded amounts of anti-CD3 mAb in 200 μl IMDM, supplemented with 5% heat-inactivated pooled human serum, 50 μM 2-mercaptoethanol, 100 IU/ml penicillin, and 100 μg/ml streptomycin. All incubations were performed in the presence of 1 μg/ml anti-CD28 mAb as a T cell costimulus (18). Without accessory cells, no T cell proliferation was observed by the anti-CD28/anti-CD3 mAb combination alone. As accessory cells, either 60,000 PMN or 2×10^5 platelets were added per well. To generate optimal T cell triggering, we first titrated PMN in anti-CD3-induced T cell proliferation experiments using an optimal amount of anti-CD3 mAb (40 ng/ml). Remarkably, the optimum for mIgG1 anti-CD3 induced proliferation was reached by supplementing ~ 30,000 PMN per well, whereas the potency of hIgG2 anti-CD3 induced T cell proliferation still increased significantly up to PMN concentrations of ~ 60,000 cells per well (observed in five experiments using cells from different donors). Since this increase of PMN-density did not affect the mIgG1 anti-CD3 induced mitogenicity, further experiments were performed using ~ 60,000 PMN per well.

Titration of platelet concentration in the presence of an optimal amounts of anti-CD3 mAb, revealed an optimal T cell proliferation at a density of ~ 2×10^5 platelets per well.

Inhibition by anti-FcγR mAb was assessed by incubation of T cells with an optimal amount of anti-CD3 mAb (40 ng/ml) in the presence of either mAb 197 (20 μg/ml), mAb IV.3 (5 μg/ml), mAb CLB Gran1 (10 μg/ml), CLB Gran1 F(ab')$_2$ fragments (20 μg/ml), or mAb 3G8 (20 μg/ml). These inhibitory mAb were preincubated for 1 h with accessory cells before purified T cells were added to the cultures. A 4-h [^{3}H]thymidine (0.2 μCi/well) incorporation assay was performed on day 4. All incubations were performed in triplicate, and SD were always < 10%.

We performed control experiments to check for (nonspecific) effects of anti-FcγR mAb on anti-CD3 induced T cell proliferation, (via potential activation and subsequent degranulation of PMN) during culture. Anti-FcγR mAb were added to an FcγR-independent T cell activation system (30). In this assay, mIgA anti-CD3 mAb were coated at high density to culture wells; a condition inducing potent proliferation of purified T cells (this mIgA mAb is a heavy chain isotype switch variant (21) of the anti-CD3 mAb used in rest of our studies). No inhibitory effects of any anti-FcγR mAb were observed in this system.

Purification of IgG paraproteins. Purified hIgG subclass proteins with kappa light chains were isolated from sera of patients with multiple myeloma by $(NH_4)_2SO_4$ precipitation and DEAE-Sephadex (Pharmacia, Uppsala, Sweden) ion-exchange chromatography. If necessary, proteins were further purified on protein A or anti-hIgG subclass Sepharose. All preparations were checked for impurities and IgG subclass content in immunoelectrophoresis, radial immunodiffusion, and hemagglutination assays (31, 32). The hIgG paraproteins used in this study were > 98% pure, as evaluated with these methods. Purified hIgG was either used in a monomeric form, after removal of aggregates by ultracentrifugation (1 h at 150,000 *g*), or as stable dimeric complexes (see below).

Dimer binding studies. Purified IgG paraproteins were incubated overnight with F(ab')$_2$ fragments of a mouse anti-human κ-light chain mAb K35, in a molar ratio of 1:1, which resulted in stable tetrameric complexes, as detailed in (24). Human monocytes (3×10^5 in 100 μl PBS/1% BSA) were incubated with 25 μl of anti-FcγR mAb 197 (7 μg/ml) and 25 μl of an optimal amount of hIgG dimers (~ 25 μg/ml) for 45 min at 4°C. The amount of mAb 197 was optimized to result in complete blockade of both monomeric IgG binding to FcγRI, and FcγRI-mediated EA-rosetting (see below). After washing, cells were subsequently incubated with FITC-conjugated F(ab')$_2$ fragments of goat anti-hIg antiserum (Cappel, Cochranville, PA), and FITC-conjugated F(ab')$_2$ fragments of rabbit anti-goat antiserum (Cappel), each

for 45 min at 4°C. Cell-bound hIgG dimers were detected flow cytometrically by a FACScan® (Becton Dickinson Immunocytometry Sys., Mountain View, CA). To remove crossreactive antibodies, all FITC-conjugates were absorbed with mIgG-coated Sepharose beads prior to use. Statistical analysis was performed using a Student's *t* test.

Dimer binding to PMN was essentially performed as detailed above, but without addition of mAb 197. Since hIgG4 dimers did not bind to FcγRII of either monocytes or PMN of any donor tested, we checked the integrity of this binding reagent by assessing dimer binding to U937 cells, which were incubated with IFN-γ (300 U/ml overnight) before use to enhance FcγRI expression (1).

EA-rosetting; specificity of inhibitory anti-FcγRI and anti-FcγRII mAb. To check for specific inhibition by anti-FcγRI and anti-FcγRII mAb in our assays, we tested these mAb in EA-rosetting experiments. Two types of indicator erythrocytes were used: rhesus-D positive human erythrocytes were optimally sensitized either with a human antiserum directed against rhesus-D (EA-hIgG; Merz and Dade, Düdingen, Switzerland), or with a mIgG1 anti-glycophorin A mAb (EA-mIgG1) (23, 33). Rosetting was scored microscopically as described before (33).

Inhibition of FcγRI- or FcγRII-mediated IgG-binding was performed by adding mAb 197 (7 μg/ml) or mAb IV.3 (2.5 μg/ml), respectively. Both antibodies were used at concentrations that inhibited specific EA-rosetting via FcγRI or FcγRII using purified monocytes and EA-hIgG or EA-mIgG1, respectively (23, 33). Rosettes were readily observed after incubation of monocytes with EA-hIgG: 70±3% of cells were scored positive (n = 3). Formation of rosettes was not inhibited by addition of mAb IV.3 (73±4% rosetting cells; n = 3), in contrast to mAb 197, which blocked EA-hIgG rosetting efficiently (2±2% rosettes; n = 3). FcγRII-mediated rosetting was measured by adding EA-mIgG1 to HR monocytes, which resulted in 58±3% rosettes (n = 3). Addition of identical concentrations of the blocking anti-FcγR mAb as above, resulted in efficient inhibition of EA-mIgG1 rosetting by mAb IV.3 (3±1% rosettes; n = 3), whereas mAb 197 did not significantly impede rosette formation (56±2% rosettes; n = 3).

We next evaluated whether FcγRI was efficiently blocked by preincubation with mAb 197 and, therefore, could be used to study specific binding to FcγRIIa on monocytes. Monocytes were first incubated for 30 min at 37°C in RPMI 1640 medium (without serum) to remove cytophilic IgG. Cells were washed twice in medium and incubated for 15 min at 4°C in PBS/0.5% BSA/0.1% NaN_3 alone, or supplemented with either anti-FcγRI mAb 197 (7 μg/ml) or mAb IV.3 (2.5 μg/ml). Subsequently, 10 μg/ml of monomeric hIgG was added, and after a 45-min incubation at 4°C, cells were washed. Cell-bound hIgG was detected flow cytometrically by a FACScan®, using FITC-conjugated $F(ab')_2$ fragments of goat anti-hIgG antiserum (Cappel). Addition of monomeric hIgG to monocytes resulted in 79±3% positive staining cells (n = 2), which was effectively blocked by preincubating the cells with mAb 197 (4±2% positive cells; n = 2), but not with mAb IV.3 (70±4% positive cells; n = 2).

Measurement of serum IgG. Sera and citrated blood samples were collected by venipuncture from 69 randomly selected volunteering colleagues, 43 males and 26 females (mean age 30±6, range 21–57 yr), and sera were stored at −20°C until used. hIgG1, hIgG2, hIgG3, hIgG4, and total hIgG concentrations were measured nephelometrically (Vlug, A., E. Nieuwenhuis, I. Knop, and R. V. W. van Eijk, manuscript in preparation). Statistical analysis was performed by a Mann-Whitney U test.

FcγRII phenotyping. The FcγRIIa phenotype of cells from different donors was analyzed by comparing the potency of mIgG1 and hIgG2 anti-CD3–mAb induced T cell proliferation in PBMC isolated from citrated blood, as detailed in (18). Briefly, 40,000 PBMC were cultured for 4 d in culture medium (see above) after addition of either mIgG1, or hIgG2 anti-CD3 mAb in an optimal concentration (40 ng/ml). As described before (18), three groups of donors could be discriminated: two groups with PBMC only responsive to either mIgG1, or hIgG2 anti-CD3 mAb, and one group with a comparable reactivity to both anti-CD3 mAb, indicating homozygous $Fc\gamma RIIa^{HR}$, homozygous $Fc\gamma RIIa^{LR}$, and heterozygous $Fc\gamma RIIa^{LR,HR}$ individuals, respectively.

In addition, FcγRIIa phenotypes of donors were assessed by using immuno fluorescence analysis on a FACScan using anti-FcγRII mAb 41H16 (which selectively reacts with $Fc\gamma RIIa^{HR}$ [34]) and mAb IV.3 (recognizing both FcγRIIa allotypic forms), followed by detection with FITC-conjugated $F(ab')_2$ fragments of goat anti-mouse antiserum (G26M17F, CLB) as described previously (18, 34). To avoid cytophilic binding of the mIgG2a mAb 41H16 to the high-affinity FcγRI, all mAb incubations were performed in the presence of 25% heat-inactivated human serum.

All experiments in this study were performed using cells isolated from either $Fc\gamma RIIa^{LR}$ or $Fc\gamma RIIa^{HR}$ homozygous donors.

Results

Human IgG dimer binding to monocytes. Human monocytes express both FcγRI and FcγRIIa constitutively (4). When used as accessory cells in anti-CD3 induced T cell proliferation, mitogenicity to chimeric hIgG1, hIgG3, and hIgG4 anti-CD3 was found to be FcγRI-mediated (18). The hIgG2 anti-CD3 mAb, however, induced FcγRIIa-mediated T cell proliferation, which was only found with PBMC of individuals expressing the $Fc\gamma RIIa^{LR}$ allotypic form (both homo- and heterozygotes) (18). We now studied binding of well-defined stable

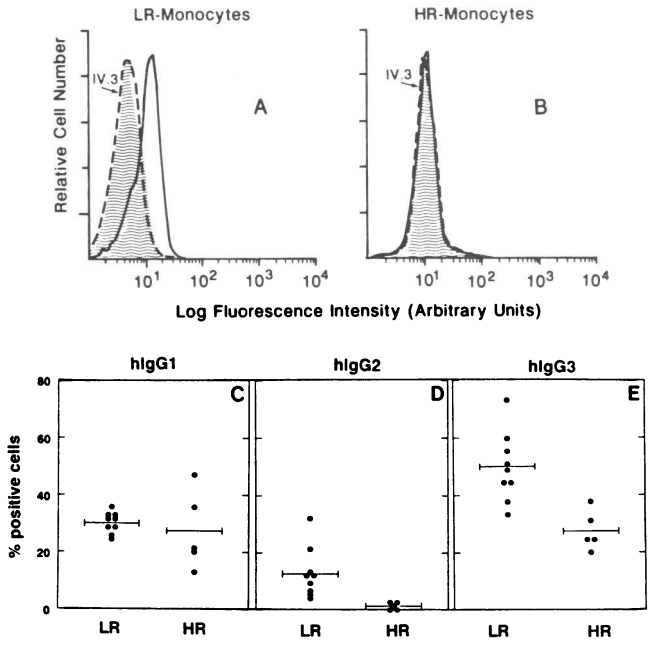

Figure 1. Binding of dimeric hIgG subclass complexes to FcγRI-blocked purified monocytes. The high affinity FcγRI on monocytes was blocked by addition of a saturating amount of inhibitory anti-FcγRI mAb 197. Stable dimeric hIgG1, hIgG2, or hIgG3 complexes were added, and binding was analyzed cytofluorometrically. In (*A* and *B*), representative experiments are shown illustrating the immunofluorescence observed after addition of hIgG2 dimeric complexes to monocytes from $Fc\gamma RII^{LR}$ and $Fc\gamma RII^{HR}$-homozygous donors, respectively. Binding was assessed after addition of hIgG2-dimers alone (*solid line*), or in the presence of mAb IV.3 (*dashed line*). Background fluorescence, observed in the absence of dimeric complexes, is indicated by shading. (*C–E*) show the binding of hIgG1, hIgG2, and hIgG3 dimeric complexes to monocytes from $Fc\gamma RIIa^{LR}$-(n = 9), and $Fc\gamma RIIa^{HR}$-typed (n = 5) homozygous donors, represented as the percentage of cells with fluorescence intensities above background levels.

dimeric hIgG complexes of the four hIgG subclasses to these cells. To allow exclusive FcγRIIa-mediated binding of hIgG dimers, we blocked the high affinity IgG Fc receptor specifically using anti-FcγRI mAb 197 (see Methods).

Purified monocytes isolated from homozygous FcγRIIa-typed donors, were incubated with dimeric complexes of defined hIgG subclasses. hIgG2 dimers were bound differentially by cells from FcγRIIa^{LR} and FcγRIIa^{HR} individuals, as illustrated in Fig. 1 *A*, and *B*, respectively. Significant binding of hIgG2 dimeric complexes was only apparent to cells bearing the FcγRIIa^{LR} allotypic form (Fig. 1 *A*), whereas the homozygous FcγRIIa^{HR}-expressing cells remained negative (Fig. 1 *B*). The same pattern of binding was observed with monocytes from nine LR and five HR donors (Fig. 1 *D*), and with four different hIgG2 myeloma proteins (data not shown). Parallel experiments were performed using hIgG1, hIgG3, and hIgG4 dimeric complexes. Both hIgG1 and hIgG3 dimers bound effectively to monocytes from the two types of donors, as shown in Fig. 1 *C*, and 1 *E*, respectively. No difference was observed in hIgG1 dimer binding between the homozygous FcγRIIa^{LR} and FcγRIIa^{HR} monocytes (Fig. 1 *C*). Cells expressing the FcγRIIa^{LR} allotypic form, however, exhibited a significantly higher capacity to bind hIgG3 dimers ($P < 0.001$; Fig. 1 *E*). In none of the experiments performed we observed binding of hIgG4 dimers to FcγRI-blocked monocytes. The percentage of cells with fluorescence above background was always < 2%, both with monocytes from FcγRIIa^{LR} (n = 15), and FcγRIIa^{HR} (n = 8) individuals. To appraise the validity of the hIgG4 dimeric complexes as binding reagents, we performed binding experiments with U937 cells (expressing FcγRI and FcγRIIa^{HR} [8]). Upon incubation of these cells with hIgG4 dimers, 99% of the U937 cells stained positive, which could be inhibited by mAb 197 (6% positive cells), but not by mAb IV.3 (99% positive cells) (n = 2). This susceptibility of hIgG4 binding to inhibition solely by anti-FcγRI mAb 197 indicates, furthermore, that FcγRIIa on U937 is incapable of interacting with hIgG4 dimers (as observed with monocytes).

The functional FcγRIIa polymorphism to hIgG2 is expressed on platelets. A substantial portion of FcγRII present on PBC is expressed on platelets. Platelets do not express other classes of FcγR (35), and seem therefore a superb model system to study FcγRII-IgG interactions. Consequently, we studied anti-CD3-induced T cell activation in the presence of platelets as accessory cells.

Fig. 2 *A* and *B* represents immunofluorescence analyses of platelets used in the T cell mitogenesis experiments shown in panels *C* and *D*, respectively. After staining with mAb IV.3, platelets derived from both donors displayed a comparable fluorescence intensity, indicating comparable FcγRIIa expression levels. Incubation of platelets with mAb 41H16 revealed similar staining as mAb IV.3 for the FcγRIIa^{HR} homozygous donor and background staining for the FcγRIIa^{LR} homozygous donor. This suggested an antigenically similar FcγRIIa polymorphism as found on monocytes and PMN.

In contrast to T cell mitogenesis studies with monocytes as accessory cells, addition of anti-CD3 mAb to platelets alone was insufficient to induce T cell proliferation. Platelets apparently lacked the ability to provide an accessory signal for full activation of T cells. We circumvented this requirement by adding an anti-CD28 mAb as costimulator to the incubations. Addition of anti-CD28 mAb alone or anti-CD28 combined with the anti-CD3 mAb CLB-T3/4 did not result in proliferation of purified T cells. Platelets, isolated from homozygous FcγRIIa^{HR} or FcγRIIa^{LR} individuals were used to study T cell proliferation induced by quantitated amounts of mIgG1 or hIgG2 anti-CD3 mAb (shown in Fig. 2 *C* and *D*). Platelets derived from an FcγRIIa^{LR} individual supported hIgG2 anti-CD3-induced proliferation efficiently, whereas mIgG1 induced proliferation was hardly detectable (Fig. 2 *C*). Platelets isolated from FcγRIIa^{HR} individuals were observed to support mIgG1 anti-CD3-induced T cell mitogenesis, but not hIgG2 anti-CD3 induced activation. Addition of mAb IV.3 to these cultures resulted in complete inhibition of both hIgG2 and mIgG1 anti-CD3-induced T cell mitogenesis (> 99% inhibition; n = 2). Platelets expressing the FcγRIIa^{LR} allotypic form, furthermore supported induction of T cell proliferation by hIgG1 anti-CD3 efficiently, but not with hIgG3, or hIgG4 (n = 2).

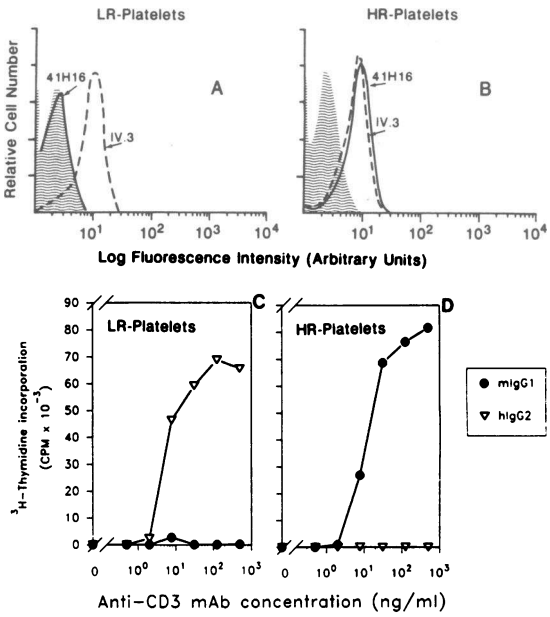

Figure 2. Characterization of platelet FcγR expression, and anti-CD3-induced T cell proliferation in the presence of platelets as accessory cells. Platelets, isolated from FcγRIIa^{LR}- (*A*) or FcγRIIa^{HR}- (*B*) homozygous donors, were incubated either with buffer alone (control; *shaded*), anti-FcγRII mAb IV.3 (*dashed line*), or anti-FcγRIIa^{HR} mAb 41H16 (*solid line*), followed by FITC-conjugated F(ab')$_2$ fragments of goat anti-mouse IgG antiserum. Platelets from these donors were subsequently used as accessory cells in mIgG1 and hIgG2 anti-CD3-induced T cell proliferation. (*C* and *D*) represent the anti-CD3-induced mitogenesis of highly purified T cells using FcγRIIa^{LR}-typed, and FcγRIIa^{HR}-typed platelets, respectively. [^{3}H]thymidine incorporation is plotted against the anti-CD3 mAb concentration. Experiments performed with platelets derived from two FcγRIIa^{LR}-, and one FcγRIIa^{HR}-homozygous donors, respectively, yielded similar results.

Interaction between (chimeric) anti-CD3 mAb and FcγR on PMN. Human neutrophils constitutively express both FcγRIIa and a GPI-linked form of FcγRIII; i.e., FcγRIIIb. FcγRIII has been implicated in modulation of FcγRII affinity and triggering (36–38). To examine this in more detail, we studied antibody-FcγR interactions on normal PMN and PMN impaired in expression of FcγRIIIb.

PMN were isolated from healthy donors homozygous for either FcγRIIa phenotype, and they were used as accessory

cells in anti-CD3–induced T cell activation experiments, as shown in Fig. 3. In these experiments, we also added anti-CD28 mAb as a T cell costimulus, since anti-CD3 mAb combined with PMN alone were incapable of inducing T cell proliferation. A dose-dependent mIgG1 anti-CD3 mAb induced T cell activation was only observed using PMN expressing FcγRIIa^{HR}, whereas hIgG2 anti-CD3 mAb preferentially induced proliferation in the presence of FcγRIIa^{LR} PMN. Cells from both types of individuals supported proliferation with hIgG1 anti-CD3, but not hIgG3 or hIgG4 subclass variants (Fig. 3). Evaluation of the role of FcγRII in these responses is complicated by expression of both FcγRIIa and FcγRIIIb on these cells. Inhibition analyses were, therefore, performed on normal and on FcγRIIIb negative (FcγRIII^{neg}) PMN.

Phenotypes of PMN used for these inhibition experiments were analyzed cytofluorometrically as shown in Fig. 4 *A* and *B*. The PMN analyzed in Fig. 4 *B*, isolated from a donor who showed a complete lack of PMN-FcγRIIIb expression caused by a genomic deletion in the FcγRIIIB locus (29), stained positive with anti-FcγRII mAb (IV.3), but not with a FcγRIIa^{HR}-specific (41H16) or anti-FcγRIII mAb (CLB Gran1). This indicated expression of the FcγRIIa^{LR} allotypic form exclusively on these cells. Fig. 4 *A* shows the analysis of a normal donor matched for expression of this FcγRIIa allotypic form. PMN of both these donors, furthermore, did not express FcγRI, as indicated by absent staining with anti-FcγRI mAb 197, and 22 (data not shown).

To evaluate which FcγR classes were involved in anti-CD3 induced T cell mitogenesis in the presence of PMN, we analyzed T cell proliferation in this system, in the presence or absence of blocking anti-FcγRI (197), anti-FcγRII (IV.3), or anti-FcγRIII (CLB Gran1) mAb. Inhibition patterns of anti-CD3 induced T cell proliferation by these anti-FcγR mAb are shown in Fig. 4 *C* and *D*. Fig. 4 *C* shows that PMN-dependent hIgG2 anti-CD3–induced T cell proliferation was completely inhibited upon addition of mAb IV.3. PMN derived from the

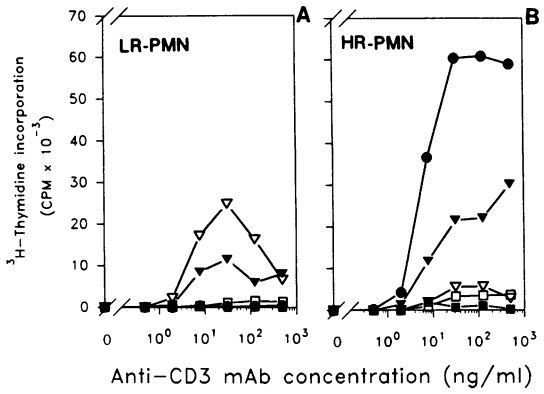

Figure 3. Induction of T cell mitogenesis by anti-CD3 isotype variants using PMN as accessory cells. Highly purified T cells were incubated with a quantitated amount of mIgG1, hIgG1, hIgG2, hIgG3, and hIgG4 anti-CD3 mAb in the presence of PMN expressing either the FcγRIIa^{LR} (*A*), or the FcγRIIa^{HR} allotypic form (*B*). Anti-CD28 mAb (1 μg/ml) was added as a costimulatory signal. [^{3}H]thymidine incorporation was plotted against the concentration of anti-CD3 mAb. The experiment shown is representative of two separate experiments with cells from the same donors. Similar results were obtained with PMN derived from 5 FcγRIIa^{LR}- and 2 FcγRIIa^{HR}-expressing donors. ●, mIgG1; ▼, hIgG1; ▽, hIgG2; □, hIgG3; ■, hIgG4.

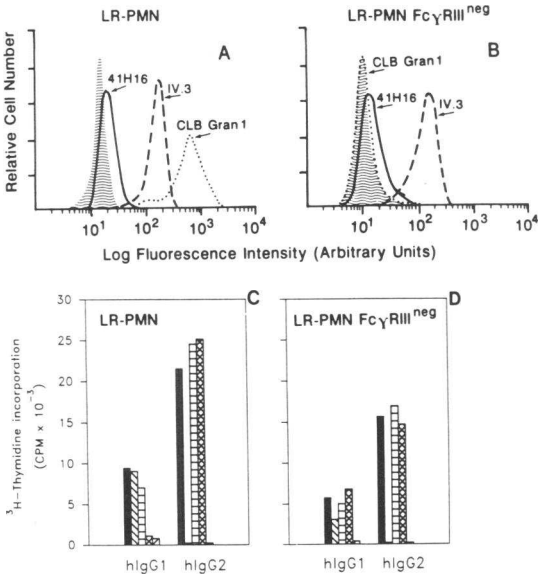

Figure 4. Characterization of FcγR-phenotype and hIgG1, and hIgG2 anti-CD3–induced T cell proliferation in the presence of normal and FcγRIII^{neg} PMN. Cytofluorometric analysis of PMN isolated from normal, and FcγRIIIb^{neg}, FcγRIIa^{LR}-homozygous individuals are shown in (*A* and *B*), respectively. PMN from both donors were incubated either with buffer alone (control; *shaded*), anti-FcγRII mAb IV.3 (which binds both allotypic forms of FcγRIIa; *dashed line*), anti-FcγRIIa^{HR}-specific mAb 41H16 (*solid line*), or anti-FcγRIII mAb CLB Gran1 (*dotted line*), followed by FITC-conjugated F(ab')$_2$ fragments of goat-anti-mouse IgG as secondary antibody. (*C* and *D*) show an analysis of hIgG1, and hIgG2 anti-CD3 induced proliferation of highly purified T cells in the presence of normal and FcγRIII^{neg} PMN, respectively. T cells were stimulated with an optimal amount of anti-CD3 mAb in medium alone (*black bars*), or in the presence of either anti-FcγRI mAb 197, anti-FcγRII mAb IV.3, anti-FcγRIII mAb CLB Gran1, or a combination of mAb IV.3 and mAb 197. (*C*) shows an experiment representative for PMN from five FcγRIIa^{LR} homozygous donors, (*D*) shows an experiment representative for two separate experiments using cells from a single FcγRIII^{neg} donor. ■, —; ▨, IV.3; ▤, 197; ■, CLB Gran1; ▨ IV.3 + 197.

FcγRIII^{neg} donor (analyzed in Fig. 4 *D*), also showed complete susceptibility of hIgG2 anti-CD3–induced T cell mitogenicity to mAb IV.3, similar to FcγRIIa^{LR}-bearing PMN from all normal donors evaluated (n = 12). In contrast, hIgG1 anti-CD3–induced T cell proliferation was strongly inhibited after blockade of FcγRIIIb (Fig. 4 *C*). Surprisingly, however, the absence of FcγRIIIb did not affect the ability of FcγRIIIb^{neg} PMN to support T cell mitogenesis induced by this hIgG subclass. The data shown in Fig. 4 *D* suggested this proliferation-induction to be partly FcγRII mediated, supported by a decrease in T cell responsiveness after addition of mAb IV.3. Remarkably, a synergistically increased inhibition was observed by combining mAb IV.3 with mAb 197. Indeed, a complication in these culture experiments may be induction of FcγRI on PMN by IFN-γ (1), produced by proliferating T cells, which may then mediate binding of hIgG1 mAb. We tested this hypothesis by analyzing cells from the cultures cytofluorometrically, using a FITC-conjugate of anti-FcγRI mAb 22, as shown in Table I. After gating on PMN, we found a significant increase of FcγRI expression after culturing the cells overnight, provided anti-CD3–stimulated T cells were present in the culture. The level of FcγRI expression on these PMN was similar to the expres-

sion level induced after culturing overnight in medium supplemented with IFN-γ ($n = 4$). In both these experiments, FcγRI-induction could be completely inhibited by addition of anti–IFN-γ mAb.

Turning our attention to FcγRIIIb, we observed a dramatic decrease in the expression level of this FcγR on PMN measured during the first 2 d of culture ($n = 3$), as indicated in Table II. The presence of soluble FcγRIII in the supernatants (data not shown) indicated that FcγRIIIb is shed from the cell membrane during culture (39). As a control, we stained cells with a (granulocyte-specific) CD66 mAb, CLB Gran10, which revealed all gated cells to be positive, without reduced fluorescence. The observation that FcγRI is rapidly induced during culture, while FcγRIIIb expression on the cell surface is decreased, might implicate a minor role for FcγRIIIb in hIgG1 anti-CD3 induced T cell mitogenesis. Expression of FcγRI, furthermore, posed a practical difficulty in that anti-FcγRIII mAb CLB Gran1 (mIgG2a subclass) may inhibit binding to FcγRI via its Fc-moiety. We, therefore, used CLB Gran1 $F(ab')_2$ fragments and mAb 3G8 (mIgG1 subclass) in inhibition analyses, which did not (CLB Gran1 $[Fab']_2$) or weakly (mAb 3G8) affect hIgG1 anti-CD3–induced T cell activation.

Human IgG dimer binding by normal and FcγRIII^{neg} PMN. We analyzed binding of dimeric hIgG subclass complexes to PMN, with normal, and absent expression of FcγRIIIb. Cells of a normal FcγRIIa^{LR} homozygous donor and the FcγRIIIb^{neg} donor described above were incubated with optimal amounts of dimers (Fig. 5). 5 *C* and 5 *D* indicate that PMN of both donors were capable of binding hIgG2 dimers, which was completely inhibitable by adding mAb IV.3. PMN of both types of donors, furthermore, bound hIgG1 and hIgG3 dimers efficiently (Fig. 5 *A, B, E,* and *F,* respectively), although the binding capacity of normal PMN for dimers of these isotypes were significantly higher. On normal PMN, the major component of this binding was FcγRIIIb-mediated, indicated by susceptibility to inhibition by anti-FcγRIII mAb 3G8 (Fig. 5 *A* and *E*). The low amount of hIgG1/hIgG3 binding observed in the presence of mAb 3G8, was decreased to background level when this mAb was combined with mAb IV.3 (data not shown; $n = 2$). The experiment with FcγRIIIb^{neg} PMN showed that, even in the absence of FcγRIIIb, PMN were still capable of binding significant amounts of hIgG1 and hIgG3. This binding, however, was now completely susceptible to blockade by mAb IV.3 (Fig. 5 *B* and *F*), and, hence, FcγRII-mediated. Binding of hIgG4 dimeric complexes to these cells was also studied, but none of the PMN preparations evaluated in our laboratory ($n = 15$), was capable of significantly binding this hIgG subclass (Fig. 5 *G* and *H*).

Table I. Induction of FcγRI on Cultured PMN

Stimuli*	% CD64-positive PMN‡§
None	7.1
IFN-γ	68.4
hIgG1 anti-CD3	7.3
hIgG1 anti-CD3 + T cells	60.5
IFN-γ + anti-IFN-γ	11.4
hIgG1 anti-CD3 + T cells + Anti-IFN-γ	9.5

* Freshly isolated PMN were cultured overnight in culture medium supplemented with the indicated agents in the presence or absence of highly purified T cells. ‡ PMN were washed and incubated with a FITC-conjugate of anti-FcγRI mAb 22. After gating on PMN, the percentage of cells displaying fluorescence intensities above background level (PMN incubated in buffer without mAb 22-FITC) was assessed. § As a control, the cells were stained with granulocyte-specific CD66 mAb CLB Gran10, which showed all gated cells to be positive.

Table II. Expression of FcγRIIIb on Cultured PMN

Days of culture*	% CD16-positive cells (mean)‡§
0	100 (1,038)
1	74 (311)
2	66 (161)

* Freshly isolated PMN were incubated in culture medium for the number of days indicated. ‡ PMN were washed and subsequently incubated with anti-FcγRIII mAb 3G8 and FITC-conjugated $F(ab')_2$ fragments of a goat anti-mouse antiserum. The percentage of cells, displaying fluorescence intensities above background level, and mean fluorescence intensities of the cell-population (between parentheses) are indicated. § As a control, PMN were stained with CD66 mAb CLB Gran10, which showed all cells to be positive.

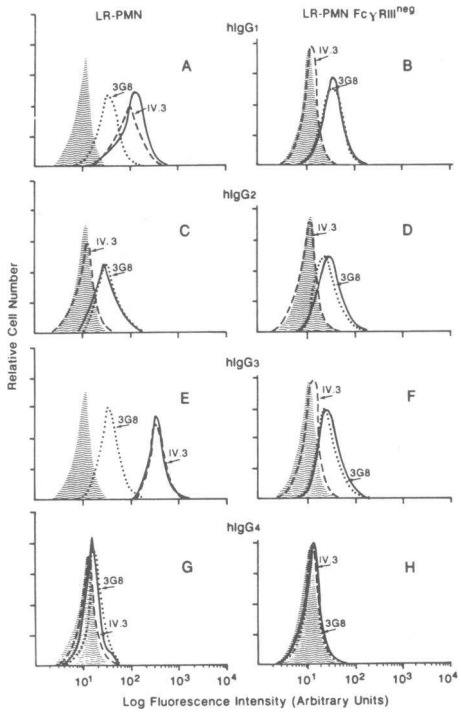

Figure 5. Binding of dimeric hIgG subclass complexes to PMN from a normal and an FcγRIII^{neg} individual. Highly purified PMN from a normal (*A, C, E,* and *G*), and a FcγRIII^{neg} (*B, D, F,* and *H*) FcγRIIa^{LR}-homozygous donor were incubated with dimeric hIgG1 (*A, B*), hIgG2 (*C, D*), hIgG3 (*E, F*), and hIgG4 (*G, H*) complexes. After washing, bound dimers were quantitated cytofluorometrically. Binding to PMN was assessed after addition of hIgG dimers either alone (*solid line*), or in the presence of anti-FcγRII mAb IV.3 (*dashed line*), or anti-FcγRIII mAb 3G8 (*dotted line*). Background fluorescence is indicated by shading. The experiment shown was repeated several times with normal ($n = 5$), and FcγRIIIb^{neg} PMN ($n = 2$) with almost identical results.

Comparing hIgG2 dimer binding to normal PMN, homozygously expressing the LR or HR allotypic form of FcγRIIa, we observed a similar pattern as on monocytes. Efficient binding was only apparent on PMN expressing FcγRIIa^{LR} (31±15% positive cells; n = 12), whereas FcγRIIa^{HR} homozygous PMN bound hIgG2 poorly (7±4% positive cells; n = 12). Incubation of PMN with GPI-PLC, which resulted in removal of ~ 90% of the GPI-linked FcγRIIIb, did not significantly affect the ability of these cells to bind hIgG2 (n = 3). hIgG2 dimer binding studies were, furthermore, performed with PMN isolated from two patients suffering from PNH, which is an acquired disorder resulting in strongly diminished expression of GPI-linked membrane proteins, such as FcγRIIIb (27, 28). PMN of both these patients were found to express FcγRIIa^{HR} only, and interacted weakly with hIgG2 dimers (resulting in 4 and 8% positive staining cells, respectively).

Correlation between FcγRIIa polymorphism and donor hIgG2 serum levels. The FcγRIIa^{LR} allotypic form seems unique in its ability to bind hIgG2, since no other hFcγR has been reported to interact efficiently with this isotype ([4, 10], this study). It has been postulated that so-called Ig binding factors (most likely soluble FcγR) may affect Ig production and proliferation of B cells (40). This prompted us to analyze hIgG subclass levels in sera of healthy donors. We randomly analyzed 69 donors, of whom 10 were found homozygous for FcγRIIa^{HR}, and 22 for FcγRIIa^{LR}. Fig. 6 *A* shows the total hIgG contents measured in the sera of these 32 homozygous donors. Comparing the two groups of individuals, we found no significant differences in total hIgG concentrations. Remarkably, a striking difference was found for hIgG2 subclass levels, which were calculated relative to the hIgG1 concentration, to correct for variations in total hIgG serum levels between different individuals (indicated in Fig. 6 *A*). As shown in Fig. 6 *B*, FcγRIIa^{LR} individuals had significantly lower hIgG2 serum levels than FcγRIIa^{HR} individuals (P = 0.02). Significant differences between the two groups were also found by calculating hIgG2 (P = 0.05) and hIgG1 (P = 0.008) levels relative to total hIgG concentration. In these analyses, hIgG2 levels were found decreased while hIgG1 levels appeared increased in FcγRIIa^{LR} versus FcγRIIa^{HR} donors. Absolute hIgG1 and hIgG2 levels, however, did not differ significantly. No significant differences were observed in hIgG3 or hIgG4 serum levels. If hIgG2 is more efficiently cleared by the LR compared to the HR allotypic form of FcγRIIa, then also heterozygous individuals might be expected to have lower relative hIgG2 subclass levels compared with homozygous FcγRIIa^{HR} donors. Comparing the hIgG2/hIgG1 ratio between the three groups, we found that this ratio (mean±SEM) of heterozygotes (0.54±0.04) was intermediate between the ratio of HR (0.67±0.05) and LR homozygotes (0.50±0.04). The difference between the heterozygotes and HR homozygotes was statistically significant (P = 0.04), whereas the difference between heterozygotes and LR homozygotes was not.

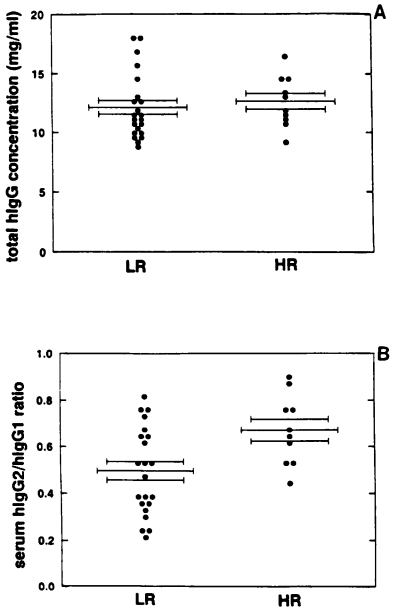

Figure 6. hIgG serum levels in FcγRIIa^{LR} and FcγRIIa^{HR} homozygous donors. Levels of hIgG1, and hIgG2 subclasses, and total hIgG were analyzed in sera of 69 randomly chosen volunteers. The serum levels of the 22 FcγRIIa^{LR}, and 10 FcγRIIa^{HR} homozygous individuals in this group are shown. (*A*) represents the total hIgG concentration measured in these sera. (*B*) shows the hIgG2 serum levels, calculated relative to the hIgG1 concentration in individuals, to correct for variations in the absolute serum hIgG levels. The mean values±SEM are indicated for each donor group.

Discussion

Recently, it was shown that the classical polymorphism of hFcγRIIa, as originally defined in mIgG1 anti-CD3-induced T cell mitogenesis (4, 11), has profound implications for hIgG2 binding. Transfected fibroblasts expressing the FcγRIIa^{LR} allotypic form were found to interact efficiently with this hIgG subclass, in contrast to FcγRIIa^{HR}-transfected cells (13). T cell mitogenesis experiments using hIgG2 anti-CD3, furthermore, indicated that differential binding of hIgG2 was inherent to normal monocytes, and was correlated with FcγRIIa phenotype (18).

In the present study we demonstrate that the functional FcγRIIa polymorphism is expressed on monocytes, PMN, and platelets. We studied this in two systems: first, by binding analyses with stable dimeric hIgG complexes, and secondly, in anti-CD3-induced T cell proliferation in which large, heavily opsonized complexes were presented to FcγR. Both in hIgG dimer binding and anti-CD3-induced T cell mitogenesis, we were able to displace hIgG2 from cells with mAb IV.3, supporting FcγRIIa-mediated binding. FcγRIIa, furthermore, bound antibodies of the hIgG1 and hIgG3, but not hIgG4 isotypes, as shown by dimer binding to FcγRI-blocked monocytes (Fig. 1). In these analyses, hIgG3 dimers were bound significantly better by FcγRIIa^{LR} than by FcγRIIa^{HR}. Since cross-talk between FcγRI and II has been observed in signalling in U937 cells (19), it seems possible that ligation of FcγRI on monocytes by mAb 197 may affect binding via FcγRII. In our opinion, this is not supported by our studies with FcγRIIa-transfected fibroblasts (13), which showed identical binding characteristics of IgG dimers, as shown with the FcγRI-blocked monocytes in the present study. Blocking of FcγRI by intact mAb 197 might cause problems resulting from cross-reactivity of its Fc region with other FcγR classes. All available FcγRI mAb, however, bind to an epitope outside the ligand-binding site (41), and only one of them (mAb 197, as used in the present study) is inhibitory by an interaction of its mIgG2a Fc region to the FcγRI-binding site ("Kurlander phenomenon" [4]). Interference in IgG dimer binding to FcγRIIa, however, seems improbable since mIgG2a has only minor affinity for this class of FcγR (4), indicated furthermore by the inability of FcγRIIa transfected fibroblasts to support mIgG2a anti-CD3-induced

T cell activation (18). Binding of dimers to FcγRII on PMN exhibited similar characteristics as on monocytes (Fig. 5). After blocking FcγRIIIb on these PMN by mAb 3G8, hIgG1 and hIgG3 dimers were still binding weakly to the cells, which could be abrogated by combining mAb 3G8 with mAb IV.3. The fluorescence intensities observed with hIgG1, hIgG2, and hIgG3 dimers to PMN on which FcγRIIIb was blocked by mAb 3G8 (Figs. 5 *A, C,* and *E*), were comparable to the staining levels observed with FcγRIII^{neg} PMN (Figs. 5 *B, D,* and *F*).

These data were in agreement with data from the anti-CD3-induced T cell proliferation studies. hIgG2 anti-CD3 mAb acted as a potent T cell mitogen in the presence of monocytes (18), platelets (Fig. 2), and neutrophils (Fig. 3), providing the FcγRIIa^{LR} allotypic form was expressed. hIgG1 anti-CD3 mAb, in contrast, induced proliferation of T cells in the presence of accessory cells from all donors, regardless of FcγRIIa-phenotype. This mitogenic activity was blocked almost completely by anti-FcγRI mAb 197 (18) or anti-FcγRIII mAb CLB Gran1 (Fig. 4 *C*) when monocytes or neutrophils were used as accessory cells, respectively. Using FcγRIIa^{LR}/FcγRIIIb^{neg} PMN as accessory cells, we also observed an induction of T cell mitogenesis by hIgG1 anti-CD3 mAb. Our observations indicate that this mitogenicity is both FcγRI- and FcγRII-mediated, suggested by low susceptibility to either mAb 197 or mAb IV.3 alone, but a synergistic inhibition by combining these anti-FcγR mAb with PMN from both types of donors. Cytofluorometric analyses of PMN during T cell mitogenesis experiments showed, furthermore, induction of FcγRI-expression on PMN, which was mediated by IFN-γ, produced by activated T cells. This is supported by inhibition of FcγRI-induction in the presence of a neutralizing anti- IFN-γ mAb. Culturing of PMN, furthermore, affected the level of FcγRIIIb expression, which was reduced dramatically. This observation, and the absent or weak inhibition by mAb CLB Gran1 F(ab')$_2$ fragments and mAb 3G8, respectively, suggest a minor role for FcγRIIIb in anti-CD3-induced T cell activation. The strong inhibition by anti-FcγRIII mAb CLB Gran1 of hIgG1 anti-CD3-induced T cell mitogenesis (Fig. 4 *C*) is, therefore, most likely mediated by the Fc-moiety of this (mIgG2a) mAb (Kurlander phenomenon [4]).

A possible explanation for the inhibition pattern of hIgG1 anti-CD3-induced T cell mitogenesis (shown in Fig. 4) may be the following: hIgG1, in first instance, most likely interacts with FcγRIIIb and FcγRIIa, supported by susceptibility of hIgG1 dimer binding on normal PMN to inhibition by mAb 3G8 and mAb IV.3, which results in activation of T cells. Production of IFN-γ by these activated T cells leads to induction of FcγRI. In parallel, FcγRIIIb is shed from the cell membrane. This implicates that these FcγR might be involved in hIgG1-induced T cell mitogenicity at different time points during the culture.

Anti-CD3-induced T cell proliferation with platelets as accessory cells indicated that the FcγRIIa polymorphism to hIgG2 was also expressed on platelets. hIgG2 anti-CD3 induced T cell proliferation was only observed in the presence of FcγRIIa^{LR} expressing cells (Fig. 2). The pattern of subclass-specific T cell mitogenesis in these experiments, in fact, appeared identical to T cell mitogenesis in the presence of FcγRIIa-transfected murine fibroblasts (18). Rosenfeld et al. (42) have shown stable interdonor variation in FcγRIIa expression levels. Differences resulting from this polymorphism were controlled for in this study, by matching different donors (two FcγRIIa^{LR}- and one FcγRIIa^{HR}-homozygous) for FcγRIIa expression levels on their platelets (Fig. 2 *A* and *B*).

Comparing the data obtained with hIgG3 in dimer-binding and T-cell mitogenicity studies, an inconsistency was found: hIgG3- and hIgG1-dimers both bound FcγR positive cells efficiently, whereas the hIgG1 chimeric mAb was superior to hIgG3 in inducing T cell mitogenesis. In the present and previous studies, we have observed that hIgG3 anti-CD3 mAb, in contrast to hIgG1 anti-CD3, were poor T cell mitogens when accessory cells from most healthy donors were used (15, 18). This was observed for two different allotypic forms of hIgG3 (15). Strikingly, we observed no differences in cytophilic binding of hIgG1 and hIgG3 anti-CD3 mAb to IFN-γ treated U937 cells (P. Parren, unpublished results). Similar observations were reported by Brüggemann et al. (43) and Steplewski et al. (44), who compared the efficacy of hIgG1 and hIgG3 chimeric mAb in antibody-dependent cellular cytotoxicity, and found a greater potency of hIgG1. Consistent with our observations, the latter study also reported this remarkable difference, in spite of a similar binding of chimeric mAb of these two subclasses to FcγR on IFN-γ treated U937 cells (44).

Summarizing the results obtained with monocytes, PMN, and platelets, the present study suggests as subclass specificity hIgG3 > hIgG1,hIgG2 ≫ hIgG4 for the FcγRIIa^{LR} allotypic form, and hIgG3,hIgG1 ≫ hIgG2 > hIgG4 for the FcγRIIa^{HR} allotypic form.

Using PMN of normal and an FcγRIII^{neg} donor, we analyzed whether cooperation between FcγRIIa and FcγRIII is taking place. Such cooperation has been described in functional studies of FcγRII-mediated phagocytosis (37), immune-complex-induced PMN actin assembly (36), and release of hydrolytic enzymes induced by IgM anti-FcγR autoantibodies (38). The question of potential cooperation between FcγR classes seems relevant in that in PNH patients who have strongly diminished expression of GPI-linked proteins, the FcγRIIIb expression is preserved (albeit at reduced level), likely because of a preferential assembly of GPI-anchors to FcγRIIIb compared to other GPI-linked membrane proteins (28). This suggests that complete FcγRIIIb deficiency may be harmful. Apart from a diseased FcγRIIIb^{neg} individual suffering from systemic lupus erythematosus (45); however, two healthy FcγRIII^{neg} individuals have recently been described who were deficient for PMN-FcγRIIIb expression, probably because of a disruption of the structural FcγRIIIB gene (29). We have now studied hIgG dimer interactions with PMN of one of these donors in detail. Effects of FcγRIIIb on IgG-binding by FcγRII, however, were not evident from our study (Figs. 4 and 5). After blockade of FcγRIIIb on normal PMN, no differences were observed between normal and FcγRIII^{neg} PMN in their ability to bind hIgG1, hIgG2, or hIgG3 dimers, which was found FcγRIIa^{LR}-mediated. Interestingly, the efficacy of hIgG1 anti-CD3-induced T cell proliferation was not affected when normal PMN were substituted by FcγRIII^{neg} cells and with both PMN populations, FcγRI and FcγRII seemed involved in sustaining T cell mitogenicity. These data raise questions about the in vivo role of the third class of FcγR, and may point to a structurally redundant system.

An important question regarding the functional FcγRIIa polymorphism remains: Is there an in vivo relevance? A study by Wee et al. (46) suggests a relevance in immunotherapy, in that differences in the efficacy of mIgG1 anti-CD8 induced clearing of T cells in kidney transplant recipients were observed

between HR and LR individuals. In the present study, we observed a significant correlation between hIgG1 and hIgG2 serum levels, and the FcγRIIa-phenotype in healthy donors. To correct for differences in total hIgG serum levels, which vary considerably between adults (47), we calculated hIgG1 and hIgG2 relative to hIgG serum levels in each individual. This seems correct since the proportion of each subclass relative to the total hIgG content is maintained within a narrow range (reviewed in [48]). Our data suggest that FcγRIIa may in some way regulate hIgG subclass production/turnover. The mechanism behind this regulation, however, remains to be elucidated. It seems unlikely that regulation occurs via FcγRII expressed on B cells, since B cells are equipped with FcγRIIb isoforms, which do not express the HR-LR polymorphism (7, 10, 34). In the murine system, modulation of Ig production and proliferation of B cells has been described by soluble FcR, collectively termed "IgG binding factors." Such factors were also demonstrated in human serum (39). Recently, molecular cloning of human FcγRIIa has generated cDNA clones lacking a transmembrane domain (8, 49), these FcγR may represent putative soluble FcγRIIa-molecules expressing the polymorphism. It will, therefore, be interesting to evaluate the possible role of soluble FcγR further with respect to the observed differences in hIgG2 serum levels found in vivo. Interestingly, hIgG2 is the main isotype induced in the humoral response after challenge with bacterial polysaccharides, and it mediates an important role in immune responses to certain capsulated bacteria, such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b. This is indicated by an increased susceptibility to these pathogens in patients with selective hIgG2 subclass deficiency (48). On the condition that these protective properties of hIgG2 antibodies are of cell-mediated nature, suggested by a low activity in C1q-binding and complement-mediated lysis (43) (although hIgG2 may be effective at high antigen densities [50]), it is important to investigate whether the FcγRIIa-phenotype is linked with susceptibility to bacterial infections.

Acknowledgments

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