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Rituximab-Dependent Cytotoxicity by Natural Killer Cells: Influence of *FCGR3A* Polymorphism on the Concentration-Effect Relationship

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ABSTRACT

The *FCGR3A* gene dimorphism generates two allotypes: FcγRIIIa-158V and FcγRIIIa-158F. The genotype homozygous for FcγRIIIa-158V (VV) is associated with higher clinical response to rituximab, a chimeric anti-CD20 IgG1 used in the treatment of B lymphoproliferative malignancies. Our objective was to determine whether this genetic association relates to rituximab-dependent cytotoxicity mediated by FcγRIIIa/CD16a+ cells. The number of CD16+ circulating monocytes, T cells, and natural killer (NK) cells in 54 donors was first shown to be unrelated to *FCGR3A* polymorphism. We then demonstrated that FcγRIIIa-158V displays higher affinity for rituximab than FcγRIIIa-158F by comparing rituximab concentrations inhibiting the binding of 3G8 mAb (anti-CD16) with VV NK cells and NK cells homozygous for FcγRIIIa-158F (FF). VV and FF NK cells killed Daudi cells similarly after FcγRIIIa engagement by saturating concentrations of rituximab or 3G8. However, the rituximab concentration resulting in 50% lysis (EC_{50}) observed with NK cells from VV donors was 4.2 times lower than that observed with NK cells from FF donors (on average 0.00096 and 0.00402 μg/ml, respectively, $P = 0.0043$). Finally, the functional difference between VV and FF NK cells was restricted to rituximab concentrations weakly sensitizing CD20. This study supports the conclusion that *FCGR3A* genotype is associated with response to rituximab because it affects the relationship between rituximab concentration and NK cell-mediated lysis of CD20+ cells. Rituximab administration could therefore be adjusted according to *FCGR3A* genotype.

INTRODUCTION

FcγRIIIa/CD16a, one of the low-affinity receptors for IgG Fc, is involved in antibody-dependent cell-mediated cytotoxicity (ADCC). It links IgG-sensitized target cells to FcγRIIIa/CD16a-bearing cytotoxic cells, *i.e.,* CD56dim natural killer (NK) cells, a fraction of monocyte/macrophages, and a fraction of T cells, and activates these effector cells. The *FCGR3A* gene, which encodes FcγRIIIa, displays a functional allelic dimorphism generating allotypes with either a phenylalanine (F) or a valine (V) residue at amino acid position 158 (1, 2). This residue directly interacts with the lower hinge region of IgG1, as recently shown by IgG1-FcγRIII cocrystallization (3, 4). Accordingly, flow cytometry studies have shown that NK cells from donors homozygous for FcγRIIIa-158V (VV) bound more human IgG1 and IgG3 than did NK cells from donors homozygous for FcγRIIIa-158F (FF; Refs. 1 and 2). This difference might result from higher affinity of the FcγRIIIa-158V allotype (2) or alternatively from higher FcγRIIIa membrane expression on NK cells expressing this allotype (5).

Rituximab (Mabthera, Rituxan) is a chimeric anti-CD20 IgG1 monoclonal antibody (mAb) consisting of human γ1 and κ constant regions linked to murine variable domains (6). Over the past few years, rituximab has considerably modified the therapeutic strategy for B lymphoproliferative malignancies, particularly non-Hodgkin's lymphomas (NHLs). Rituximab, alone or in combination with chemotherapy, has been shown to be effective in the treatment of both low-intermediate and high-grade NHLs (7–12), although the response rate varies with the histological type, with low-grade/follicular lymphomas displaying the best response rate (8, 10, 11). Nevertheless, 30–50% of patients with low-grade NHLs exhibit no clinical response, and the actual causes of treatment failure remain largely unknown.

In vitro studies suggest that rituximab induces lymphoma cell lysis through ADCC (6, 13), complement-dependent cytotoxicity (6, 13–16), FcγRII/CD32-dependent phagocytosis (13), or direct signaling leading to apoptosis (17–19). The implication of both FcγR and complement activation in the *in vivo* antitumor effect of rituximab against CD20+ lymphoma cell lines has been clearly demonstrated in murine models (20, 21). We have recently shown an association between *FCGR3A* genotype and response to rituximab in previously untreated follicular NHL patients with low tumor burden (22). Indeed, VV patients had a higher probability of experiencing a clinical response compared with F carriers. The association between *FCGR3A* genotype and clinical response to rituximab has also been observed in relapsed follicular NHL patients (23) and in Waldenström's macroglobulinemia patients (24). In addition, systemic lupus erythematosus patients with the VV or VF genotype had better B-cell depletion after rituximab treatment than FF patients (25). Although the genetic association does not demonstrate that the mode of action of rituximab involves FcγRIIIa, we postulated that VV patients show a better response because they have a more efficient rituximab-dependent ADCC against CD20+ cells. The ADCC efficiency may depend on variability of target cells and/or effector cells. However, lymphoma cells from patients with distinct histological types expressing different levels of CD20 are equally sensitive to ADCC in the presence of rituximab (13). The aim of this study was therefore to investigate the influence of the *FCGR3A* polymorphism on rituximab-dependent ADCC mediated by CD16+ effector cells.

We first investigated the number of circulating CD16+ mononuclear cells in relation to *FCGR3A*-158V/F polymorphism in 54 blood donors. We then compared the affinity of the two allotypic forms of FcγRIIIa for rituximab. Finally, we studied the influence of *FCGR3A*-158V/F polymorphism on the relationship between rituximab concentration and rituximab-dependent NK cell-mediated cytotoxicity.

MATERIALS AND METHODS

mAbs. 679.1 Mc7 control irrelevant mAb, unconjugated and FITC-conjugated 3G8 mAb specific for CD16, unconjugated T199 and phycoerythrin-conjugated NKH-1 mAbs specific for CD56, and phycoerythrin-cyanin 5.1-conjugated UCHT1 mAb specific for CD3 were purchased from Beckman Coulter (Villepinte, France). FITC-conjugated DJ130c mAb specific for CD16 was obtained from Dako (Trappes, France). All of these mAbs are mouse

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IgG1. FITC-conjugated goat antihuman IgG F(ab′)$_2$ was purchased from Menarini (Antony, France).

Cell Culture. Daudi cells were cultured in 75-cm^2 tissue culture flasks (Falcon 3024; Becton Dickinson Labware Europe, Le Pont De Claix, France) at 37°C in 5% CO_2 humidified air. Cells were grown in culture medium: RPMI 1640 (Eurobio, Les Ulis, France) supplemented with 10% heat-inactivated FCS (Invitrogen SARL, Cergy Pontoise, France); 2 mM L-glutamine (Bio Whittaker Europe, Verviers, Belgium); 1 mM sodium pyruvate (Invitrogen); 50 units/ml penicillin; and 50 μg/ml streptomycin (Bio Whittaker Europe).

Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and Preparation of Peripheral Blood Lymphocytes, Monocytes, T Cells, and NK Cells. PBMCs and peripheral blood lymphocytes were prepared from the peripheral venous blood of blood donors by Lymphoprep (AbCys S.A., Paris, France) centrifugation as described previously (26). Monocytes and T cells were positively selected using MACS CD14 and MACS CD3 MicroBeads (Miltenyi Biotec, Paris, France), respectively, and NK cells were negatively selected using MACS NK Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's recommendations. The labeled cells were retained on a MACS column in the magnetic field of a VarioMACS separator (Miltenyi Biotec).

Preparation of Target Cells. Daudi cells were washed and resuspended in RPMI 1640. Cells (10×10^6) were labeled for 90 min with 100 μCi of $Na_2{}^{51}CrO_4$ (DuPont-NEN, Les Ulis, France) at 37°C in 5% CO_2, washed three times in RPMI 1640, and incubated for 1 h with culture medium to allow spontaneous release. Finally, ^{51}Cr-labeled cells were washed twice and resuspended in culture medium, and 2×10^4 cells/well were added to 96-well round-bottomed plates.

Cytotoxicity Assay. Cytotoxicity assay was performed as described previously (26). Rituximab or 3G8 mAb at the indicated final concentration was added to the ^{51}Cr-labeled target cells immediately before adding effector cells. Each assay was set up in triplicate, and the results were expressed as the percentage of specific lysis: (experimental cpm − spontaneous cpm) × 100/(maximum cpm − spontaneous cpm).

Analysis of the Relationship between Rituximab Concentration and Daudi Cell Lysis. The concentration-effect relationship of rituximab-dependent cytotoxicity by NK cells was analyzed for each individual with an Emax model, using WinNonLin 3.1 (Pharsight, Mountain View, CA), as follows:

$E = E0 + (Emax \times C)/(EC_{50} + C)$ where E is the effect (lysis), E0 is basal lysis in the absence of rituximab (effector cells alone), Emax is maximum lysis induced by rituximab, C is rituximab concentration, and EC_{50} is the concentration of rituximab leading to 50% of Emax. Pharmacodynamic parameters were compared with a nonparametric Mann-Whitney test, using Statistica 5.5 A (StatSoft, Maisons-Alfort, France). Results were considered significant if $P < 0.05$. E/Emax percentage at a given rituximab concentration was calculated as follows: % = 100 × predicted lysis at given rituximab concentration/Emax.

Enumeration of Circulating CD16+ PBMCs. Phenotypic analysis of CD16+ PBMCs was performed according to a standard no-wash whole-blood procedure using a PrepPlus workstation (Beckman Coulter). Blood samples (100 μl) were incubated with 20 μl of FITC-conjugated anti-CD16, 5 μl of phycoerythrin-conjugated anti-CD56, and 20 μl of phycoerythrin-cyanin 5.1-conjugated anti-CD3 mAbs for 15 min at 18–20°C. RBC lysis and cell fixation were performed using a TQ-Prep workstation and ImmunoPrep reagent system (Beckman Coulter) following the manufacturer's recommendations. Cells were analyzed using an EPICS-XL-MCL flow cytometer (Beckman Coulter) as described previously (27).

Binding of Rituximab to Daudi Cells. Daudi cells (5×10^5) in PBS were incubated with varying concentrations of rituximab for 30 min at 4°C. After two washes with PBS, cells were incubated (30 min at 4°C) with FITC-conjugated goat antihuman IgG F(ab′)$_2$. After two washes with PBS, cells were analyzed by flow cytometry. The percentage of CD20 occupancy on Daudi cells at a given rituximab concentration was calculated as follows: % = 100 × (MFI at given rituximab concentration − MFI in absence of rituximab)/(MFI at 0.2 μg/ml rituximab − MFI in absence of rituximab), where MFI is mean fluorescence intensity.

Rituximab-Binding Properties of FcγRIIIa. NK cells (15×10^4) were incubated with varying concentrations of rituximab (30 min, 4°C). Cells were then incubated with FITC-conjugated 3G8 (0.1 μg/ml) and phycoerythrin-conjugated anti-CD56 mAb (30 min, 4°C), washed twice in PBS at 4°C, and analyzed by flow cytometry. Results were expressed as the percentage of inhibition of 3G8 mAb binding: (MFI in absence of rituximab − MFI in presence of rituximab) × 100/(MFI in absence of rituximab). HPLC analysis of rituximab preparation was performed periodically to ensure maintenance of the monomeric IgG. In addition, the lack of binding of rituximab preparation to polymorphonuclear cells was verified periodically by flow cytometry (5).

***FCGR3A*-158V/F Genotyping.** Genotyping of the *FCGR3A*-158V/F polymorphism was performed using a single-step multiplex allele-specific PCR and fluorescence melting curve analysis assay as described previously (28).

Statistical Analysis. Differences between the three genotypes were compared by Kruskal-Wallis nonparametric ANOVA test followed by Mann-Whitney tests. Within-genotype differences in 3G8 binding were compared by Wilcoxon paired nonparametric test. Statistica 5.5 A (StatSoft) was used, and results were considered significant if $P < 0.05$.

RESULTS

Circulating CD16+ Mononuclear Cells in *FCGR3A*-158V/F Genotyped Donors. The number of peripheral blood CD16+ mononuclear cells, CD16+ monocytes, CD3+CD16+ T cells, and CD3-CD16+ NK cells were analyzed by flow cytometry in 54 blood donors simultaneously tested for the *FCGR3A*-158V/F polymorphism. Of the 54 donors, 7 and 21 were homozygous for *FCGR3A*-158V and *FCGR3A*-158F, respectively, and 26 were heterozygous (Table 1). The three groups were not different in terms of sex or age.

Table 1 *Enumeration of circulating CD16+ mononuclear cells according to FCGR3A genotype*[a]

	Circulating mononuclear cell (cells/μl)			
	All samples (n = 54)	F/F samples (n = 21)	V/F samples (n = 26)	V/V samples (n = 7)
Total PBMCs	2630 ± 530[b]	2540 ± 410	2700 ± 610	2640 ± 590
CD16+ PBMCs	362 ± 190	361 ± 185	364 ± 211	357 ± 138
Total Monocytes	457 ± 136	446 ± 112	476 ± 157	420 ± 121
CD16+ Monocytes	43 ± 20	40 ± 18	45 ± 21	44 ± 23
	(28.1 ± 5.0)[c]	(25.0 ± 3.8)[d]	(29.7 ± 5.1)[d,e]	(31.5 ± 1.8)[e,f]
Total Lymphocytes	2130 ± 450	2060 ± 310	2160 ± 520	2210 ± 560
CD3+CD16+ Lymphocytes	35 ± 31	26 ± 20	39 ± 36	44 ± 34
	(4.5 ± 2.5)	(4.7 ± 2.8)	(4.3 ± 2.6)	(4.0 ± 0.9)
CD3-CD16+ Lymphocytes	279 ± 166	292 ± 176	271 ± 174	267 ± 117
	(20.3 ± 4.3)	(16.6 ± 2.5)	(22.5 ± 3.3)[e]	(23.2 ± 2.9)[g]

[a] Phenotypic analysis of PBMC was performed according to a standard no-wash whole-blood procedure, with a combination of anti-CD16, anti-CD56, and anti-CD3 mAbs, followed by flow cytometry analysis. Gates were set on PBMC, monocytes, and lymphocytes with forward and side scatters. The *FCGR3A*-158V/F genotyping was performed as described previously (28).

[b] Results are expressed as the mean ± SD of cells/μl.

[c] Results in brackets are the mean ± SD of CD16 staining with 3G8 mAb, expressed as MFI arbitrary units.

[d] $P < 0.001$ *versus* MFI of CD3-CD16+ lymphocytes with the same genotype.

[e] $P < 0.001$ *versus* MFI of CD16+ cells from FF donors (within the cell population).

[f] $P < 0.02$ *versus* MFI of VV CD3-CD16+ lymphocytes.

[g] $P < 0.01$ *versus* MFI of FF CD3-CD16+ lymphocytes.

When all samples were analyzed together, we found that 13.8% of PBMCs, 9.4% of monocytes, and 14.7% of lymphocytes were CD16+. The vast majority of CD16+ PBMCs were NK cells (77.1%), whereas CD16+ monocytes and CD3+CD16+ lymphocytes represented 11.9% and 9.7% of these cells, respectively. No difference was found among the three genotype groups in terms of number of circulating CD16+ mononuclear cells, CD16+ monocytes, CD3+CD16+ T cells, and CD3-CD16+ NK cells (Table 1). The level of CD16 expression on monocytes was slightly higher than that observed on NK cells whatever the genotype, whereas it was very low on CD3+CD16+ T cells. Finally, higher binding of anti-CD16 3G8 mAb to NK cells and monocytes was observed in *FCGR3A*-158V homozygous and heterozygous donors compared with homozygous *FCGR3A*-158F donors (Table 1). Given that the binding of several other anti-CD16 mAbs is similar on NK cells from VV and FF donors (2), it is likely that the difference in binding of 3G8 reflects a greater affinity of this mAb for the V allotype.

Influence of *FCGR3A*-158V/F Polymorphism on Rituximab-Binding Properties of NK Cell FcγRIIIa. It has been assumed that the *FCGR3A*-158V/F polymorphism affects FcγRIIIa affinity for IgG1 (2). To address this question, we compared the ability of rituximab (IgG1) to inhibit the binding of FITC-conjugated 3G8 mAb (29) with NK cells from VV and FF donors. A 50% inhibition of 3G8 mAb binding to VV and FF NK cells was achieved with less than 0.15 mg/ml and more than 0.8 mg/ml rituximab, respectively (Fig. 1). The difference in the ability of rituximab to block 3G8 mAb binding to FcγRIIIa on VV and FF NK cells cannot result from a difference in the affinity of 3G8 mAb binding to FcγRIIIa allotypes. Indeed, the higher binding of 3G8 mAb to VV NK cells (Table 1) should lead to less rather than more blockade of 3G8 mAb binding in the presence of rituximab. These findings thus indicate that FcγRIIIa expressed on NK cells from VV donors binds rituximab with higher affinity than FcγRIIIa expressed on NK cells from FF donors.

Binding of Rituximab to Daudi Cells. To study the *in vitro* susceptibility of B cells to ADCC in the presence of rituximab, Daudi cells (HLA class I negative and CD20 positive) were used as target cells. To study the binding of rituximab, Daudi cells were incubated with rituximab concentrations ranging from 0.0002 to 2 μg/ml followed by FITC-conjugated antihuman IgG $F(ab')_2$ that did not recognize the membrane IgM on Daudi cells (Fig. 2). Weak binding of rituximab was detected with 0.0006 μg/ml rituximab. Binding then increased dramatically with increasing concentrations reaching a maximum at 0.2 μg/ml.

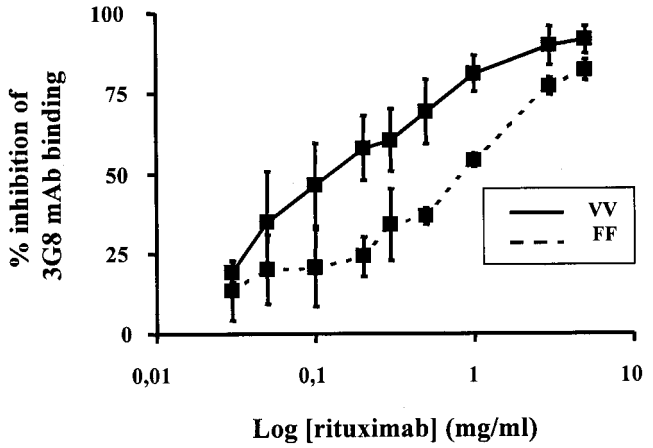

Fig. 1. Binding of rituximab to NK cells from *FCGR3A*-158 VV and FF donors. Purified NK cells from homozygous VV and FF blood donors were incubated with varying concentrations of rituximab for 30 min at 4°C followed by FITC-conjugated anti-CD16 3G8 mAb and then analyzed by flow cytometry. Percentages of inhibition of 3G8 binding were calculated as described in "Materials and Methods," and the results are expressed as the mean ± SD (n = 3 VV and 3 FF).

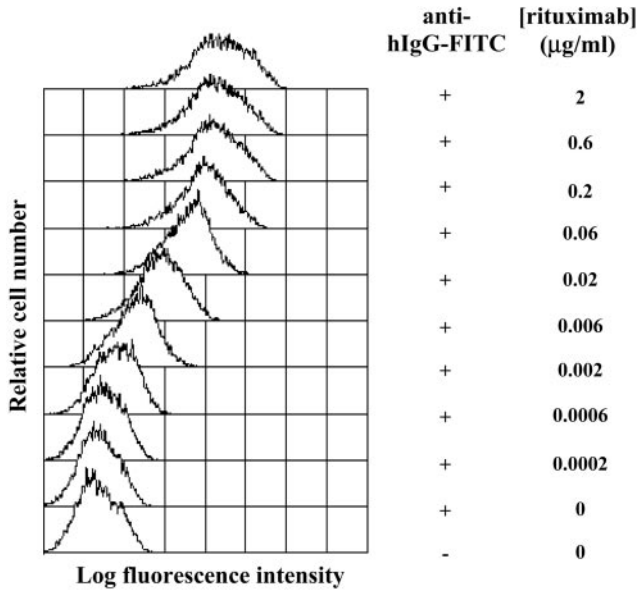

Fig. 2. Binding of rituximab to Daudi cells. Daudi cells were incubated with varying concentrations of rituximab followed by FITC-conjugated goat antihuman IgG $F(ab')_2$ and then analyzed by flow cytometry. Fluorescence intensity is displayed on the *X* axis (in log scale) and cell number on the *Y* axis (results are from one representative experiment among three).

Cytolytic Potential of NK Cells from VV and FF Donors in Response to Optimal FcγRIIIa Stimulation. As expected, Daudi cells were resistant to lysis in the absence of rituximab but were killed efficiently by PBMCs and peripheral blood lymphocytes in the presence of 0.2 μg/ml rituximab (not shown). In addition, lysis did not increase in the presence of 20 μg/ml rituximab, and Daudi cells were not killed by purified monocytes or by purified T cells, but they were killed very efficiently by purified NK cells in the presence of rituximab (not shown). We next addressed the question of the influence of *FCGR3A* polymorphism on the cytotoxic response of NK cells to optimal FcγRIIIa engagement. Daudi cells express CD32/FcγRII, which binds mouse IgG1 (30). They may therefore be used as targets in a redirected killing assay in the presence of anti-CD16 3G8 mAb. Daudi cells were resistant to lysis in the presence of anti-CD56 T199 mAb (data not shown), whereas they were killed very efficiently in the presence of 3G8 mAb (Fig. 3). Although 3G8 mAb bound slightly more efficiently to NK cells from VV donors (Table 1), the lysis observed with VV and FF NK cells in the presence of 3G8 mAb was similar and was also equivalent to that observed in the presence of saturating amounts of rituximab (Fig. 3).

Influence of *FCGR3A*-158V/F Polymorphism on the Concentration-Effect Relationship of Rituximab-Dependent NK Cell-Mediated Cytotoxicity. The influence of the *FCGR3A*-158V/F genotype on the lysis of Daudi cells was then analyzed in the presence of decreasing concentrations of rituximab (0.2 to 0.0002 μg/ml), with NK cells from six VV and six FF donors (E:T ratio = 2.5:1). As expected, for each donor, the observed lysis of Daudi cells increased with increasing concentrations of rituximab and reached a plateau at high concentrations (Fig. 4). The relationship between Daudi cell lysis and rituximab concentrations was analyzed for each individual using an Emax model. Basal lysis and maximal lysis induced by rituximab with NK cells from VV and FF donors were not different: E0 values obtained with VV and FF NK cells were 11.3 ± 9.9% and 8.3 ± 3.5% specific lysis, respectively, P = 1; whereas Emax values were 47.4 ± 6.2% and 41.9 ± 9.5% specific lysis, respectively,

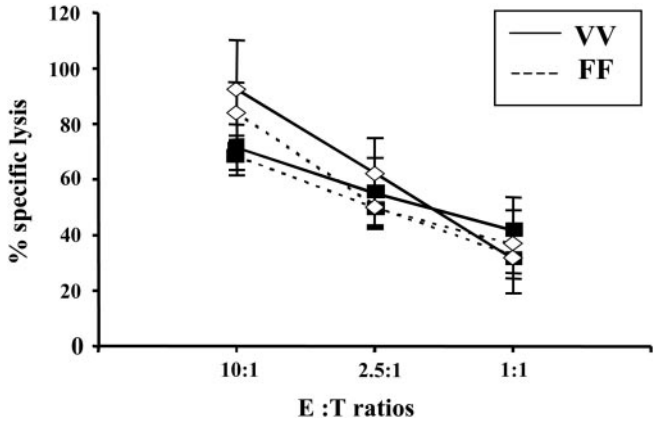

Fig. 3. Lysis of Daudi cells by NK cells from VV and FF donors after optimal FcγRIIIa stimulation. ^{51}Cr-labeled Daudi cells were incubated for 4 h at 37°C with NK cells from homozygous VV (*solid lines*) and FF (*dotted lines*) donors in the presence of 0.2 μg/ml rituximab (◇) or 0.2 μg/ml 3G8 mAb (■). Cytotoxicity against Daudi cells is expressed as the mean ± SD percentage of specific lysis (n = 6 VV and 6 FF)

P = 0.3939. By contrast, the rituximab concentration resulting in 50% lysis of target cells obtained with NK cells from VV donors was on average 4.2 times lower than that obtained with NK cells from FF donors: EC_{50}s obtained with VV and FF NK cells were 0.00096 ± 0.00058 and 0.00402 ± 0.00236 μg/ml, respectively, P = 0.0043.

DISCUSSION

We and others (22–25) have recently shown an association between the *FCGR3A* genotype and the clinical response to rituximab. We postulated that the better response to rituximab observed in *FCGR3A*-158V homozygous patients may be related to better ADCC against CD20+ cells (22). Because rituximab-mediated ADCC may first depend on the number of CD16+ effector cells in a given patient, peripheral blood CD16+ effector cells were enumerated in 54 *FCGR3A*-genotyped blood donors. No difference in the numbers of circulating CD16+ PBMCs, CD16+ monocytes, CD16+ T cells, and CD16+ NK cells was found among the three groups of donors, showing that the number of circulating CD16+ effector cells is unrelated to the *FCGR3A*-158 polymorphism.

A human CD20-expressing target cell was required to study the influence of *FCGR3A*-158 polymorphism on the rituximab-dependent CD16+ effector cell-mediated cytotoxicity *in vitro*. The Burkitt lymphoma cell line Daudi was chosen for several reasons, although Daudi cells are not representative of NHL cells. First, Daudi cells are resistant to lysis by effector cells in the absence of antibodies, and rituximab binds efficiently to them (Fig. 2). Second, they express CD32/FcγRII and therefore can be used as target cells in a redirected killing assay. Finally, the fact that they are HLA-class I negative (31) allows comparison of the cytolytic activities of effector cells from different donors, independently of the HLA-specific inhibitory receptors expressed on these effectors. In agreement with a recent study (32), we found that Daudi cells were not killed by purified monocytes or T cells in the presence of a saturating concentration of rituximab (not shown), whereas they were killed very efficiently by NK cells. These results suggest that NK cells are important effector cells in the mechanism of action of rituximab, although they do not rule out the possibility that other CD16+ effector cells, especially activated macrophages, could mediate rituximab-dependent cytotoxicity *in vivo*. Analysis of the concentration-effect relationship of rituximab-dependent NK cell-mediated cytotoxicity showed that the EC_{50} obtained with NK cells from VV donors was 4.2 times lower than that obtained with NK cells from FF donors, whereas Emax and E0 were not different. These results are in accordance with the previous observation that human low-IgG-binding NK cells were able to lyse chicken erythrocytes better than NK cells from high-IgG-binding NK cells in the presence of low concentrations of rabbit antichicken erythrocyte IgG (5). Shields *et al.* (33) have shown that some substitutions in the Fc sequence improved both the binding of monomeric human anti-IgE to FcγRIIIA-transfected Chinese hamster ovary cells and Herceptin-mediated ADCC of SKBR-3 cells by NK cells. These effects were more pronounced on the FcγRIIIA-158F than on the FcγRIIIA-158V allotype. In addition, it was shown that the more the substitutions improved binding to FcγRIIIA, the more they increased ADCC, whatever the genotype. Finally, the differences in ADCC were observed in the presence of a concentration of Herceptin variant (2 ng/ml) that is very close to the EC_{50} observed in our study. Thus, our results are consistent with these findings. Interestingly, it can be calculated from the results shown in Fig. 2 and Fig. 4 that less than 5% and 15% of CD20 molecules on Daudi cells are bound by rituximab at the EC_{50}s obtained with VV and FF NK cells, respectively. In addition, rituximab concentrations yielding more than 40% occupancy of CD20 (*i.e.*, above 0.02 μg/ml) were sufficient to obtain more than 80% of maximum lysis, whatever the genotype. Thus, the increased ADCC associated with expression of the FcγRIIIa-158V allotype on NK cells was restricted to a rituximab concentration range weakly sensitizing CD20 on Daudi cells. We therefore conclude that both the level of opsonization and the 158V/F FcγRIIIa polymorphism influence the killing of Daudi cells.

The fact that VV and FF NK cells killed Daudi cells similarly after optimal FcγRIIIa engagement by saturating concentrations of rituximab or by 3G8 anti-CD16 mAb and respond by an indistinguishable rise in Ca^{2+} to 3G8 (2) shows that the two FcγRIIIa allotypes are not different in terms of intracellular signaling and cytolytic potential. Thus, the functional difference between the two allotypes that we demonstrated at low concentrations results primarily from their difference in IgG1 binding. Flow cytometry studies have shown that human IgG1 binds more strongly to VV NK cells than to FF NK cells (1, 2, 5). Based on titration of IgG binding to NK cells from low- and high-IgG-binding individuals, Vance *et al.* (5) concluded that the absolute number of FcγRIIIa receptors rather than FcγRIIIa affinity accounts for the difference in IgG binding. Conversely, the fact that

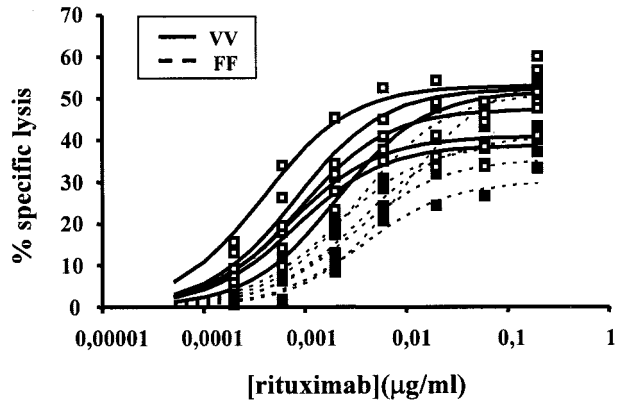

Fig. 4. Relationship between rituximab concentration and Daudi cell lysis by NK cells. ^{51}Cr-labeled Daudi cells were incubated for 4 h at 37°C with NK cells from homozygous VV and FF donors (E:T ratio = 2.5:1) in the presence of varying concentrations of rituximab. Cytotoxicity against Daudi cells is expressed as the mean percentage of (lysis in presence of rituximab − basal lysis in absence of rituximab) of each triplicate (□, VV; ■, FF) and as predicted by the Emax model (see "Materials and Methods"; *solid lines*, VV, n = 6; *dotted lines*, FF, n = 6). Observed and model-predicted basal lysis (E0), which was not different between VV and FF, was subtracted to display rituximab-dependent lysis.

similar fluorescence intensities were observed with several anti-CD16 mAbs on NK cells from VV and FF donors led Wu *et al.* (2) to propose that these cells express similar levels of FcγRIIIa and that 158-V/F polymorphism affects FcγRIIIa affinity. However, we observed a significantly higher binding of 3G8 anti-CD16 mAb on NK cells and monocytes from VV and VF donors compared with FF donors in accordance with previous reports (1, 5). Conversely, the binding of the DJ130 anti-CD16 mAb is higher on NK cells from FF and VF donors than on those from VV donors.[6] These conflicting results show the important limitations in the use of anti-CD16 mAbs and flow cytometry to conclude on the levels of FcγRIIIa expression. However, our results (Fig. 1) unambiguously demonstrate that FcγRIIIa-158V has a higher affinity for rituximab—and probably for all human IgG1 including therapeutic recombinant mAbs—than FcγRIIIa-158F.

The rituximab-depleting effect observed *in vivo* is the result of different complementary mechanisms including complement-dependent cytotoxicity (6, 13–16), apoptosis (17–19), phagocytosis, and ADCC (6, 13). The implication of both FcγR and C1q in the *in vivo* antitumor effect of rituximab against CD20+ lymphoma cell lines has been clearly demonstrated in murine models (20, 21), suggesting that ADCC and complement activation are essential for rituximab therapeutic effect. In addition, the fact that opsonization of target cells with complement components results in increased lysis by NK cells that express the CD11b/CD18 receptor (34, 35) strongly suggests that ADCC and complement activation may have a cooperative activity *in vivo* (32, 36). Complement-dependent cytotoxicity is mainly dependent on lymphoma cell variability, especially on both their CD20 and complement regulatory protein membrane expression (13, 14, 16), although response to rituximab therapy has been shown to be independent of the level of expression of these proteins on the tumor tissue (37). These parameters have no detectable influence on apoptosis, phagocytosis, or ADCC (13). By contrast, the present study is the first to show that rituximab-mediated ADCC is dependent on the allotypic form of FcγRIIIa expressed by NK cells. Therefore, variability at both the tumor cell level and the patient's immune system level may influence the response to rituximab, in addition to the tumor burden at the time of treatment (10) and rituximab concentrations obtained in patients (8, 38).

In agreement with results obtained with Raji and WIL.2-S B-cell lines (39, 40), lysis of Daudi cells was detected with concentration around 0.0001 μg/ml rituximab and reached a maximum at around 0.1 μg/ml, whatever the genotype. The difference in the cytotoxic response between VV and FF NK cells was observed over the 0–0.02 μg/ml range. On the other hand, serum rituximab concentrations observed *in vivo* range from around 1000 μg/ml for peak concentrations to around 20 μg/ml several weeks after ending the infusions (38, 41), raising the question of the clinical relevance of our *in vitro* result. However, rituximab concentrations at the site of action (mainly in lymphadenopathies) may be different from those measured in the blood. In addition, for anticancer agents, the duration of drug exposure above a defined threshold concentration is sometimes more important than the extent of the exposure (42, 43). Using the data presented by Berinstein *et al.* (38), it can be calculated that responders have a longer half-life than nonresponders (12 *versus* 8 days) and that the former group has been exposed to concentrations above 0.0001 μg/ml (*i.e.*, the concentration at which ADCC was detected *in vitro*) during 20 months *versus* 13 months for nonresponders. It has also been reported that some NHL patients have delayed clinical response (between day 78 and month 12 after rituximab infusion; Ref. 10). This observation shows that long-lasting effector mechanisms are involved in the therapeutic effect of rituximab and suggests that the difference between VV and FF NK cells observed in our *in vitro* model may be clinically relevant. Thus, the present study supports the conclusion that the *FCGR3A* genotype is associated with the therapeutic action of rituximab because it affects the relationship between rituximab concentration and B-cell lysis by NK cells. The consequence of the present findings is that rituximab dose or administration schedule may be adjusted in FF patients to ensure a longer exposure to effective concentrations to obtain a better clinical response. More generally, such a pharmacogenetic approach has to be taken into account to improve therapeutic efficacy of cytolytic mAbs in addition to methods that have been shown to enhance NK cell response to tumor cells, such as engineering the Fc portion to increase mAb binding to FcγRIIIa (32, 44) or stimulating NK cells by cytokines (35, 45, 46).

[6] S. Dall'Ozzo, S. Tartas, G. Paintaud, G. Cartron, P. Colombat, P. Bardos, H. Watier, and G. Thibault, unpublished observations.

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