Involvement of the High-Affinity Receptor for IgG (FcyRI; CD64) in Enhanced Tumor Cell Cytotoxicity of Neutrophils During Granulocyte Colony-Stimulating Factor Therapy

By Thomas Valerius, Roland Repp, Ton P.M. de Wit, Susanne Berthold, Erich Platzer, Joachim R. Kalden, Martin Gramatzki, and Jan G.J. van de Winkel

Three different classes of Fc receptors for IgG (FcyRI) are currently distinguished in humans, of which polymorphonuclear phagocytes (PMN) normally express both low-affinity receptor classes—FcyRII (CD32) and FcyRIII (CD16). During therapy with granulocyte colony-stimulating factor (G-CSF), neutrophils from patients with various malignancies and different hematologic disorders were found to additionally express high levels of the receptor with high affinity for IgG (FcyRI; CD64). For these patients, the relative fluorescence intensity (rFI) for FcyRI was 5.3 (range, 1.7 to 10.3; n = 19), compared with 1.0 (range, 1.0 to 1.1; n = 8) for healthy donors. The expression of FcyRI during G-CSF therapy could be confirmed by using a panel of six CD64-specific antibodies, and by showing mRNA for FcyRI. So far, three genes for FcyRI have been identified, encoding four distinct transcript products. By reverse transcriptase-polymerase chain reaction technology, transcripts for both membrane-associated isoforms (hFcyRIa and hFcyRIb2) could be detected. The functional activity of FcyRI on PMN during G-CSF therapy was shown by measuring binding of monomeric human IgG and antibody-dependent cellular cytotoxicity (ADCC). Thus, FcyRI-positive neutrophils displayed enhanced ADCC activity to glioma (A1207), squamous cell (A431), and ovarian (SK-oV3) carcinoma cell lines. The involvement of FcyRI in this increased cytotoxic activity was shown by blocking Fcy receptors with monoclonal antibodies, and by using Fab specific antibodies with specificities against tumor-related antigens and FcyRI, resulting in solely FcyRI-mediated cytotoxicity. Therapeutically, this additional Fc receptor on PMN may increase the efficacy of experimental antibody therapy.

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FOR THE DEFENSE against invading microorganisms, a complex network of humoral and cellular components exists. Within this system, receptors for the constant part of Igs (Fc receptors) play a central role connecting the specific humoral immune response to the phagocyte compartment. By molecular and functional analysis, three distinct classes of Fc receptors for IgG can be distinguished: one with high affinity for IgG (FcyRI; CD64) and two classes that interact well only with complexed IgG (FcyRII, CD32; and FcyRIII, CD16). Eight different genes have been identified to encode the members of these FcR classes. All genes have been located to the long arm of chromosome 1: FcyRI on 1q21,13 and FcyRII and FcyRIII on 1q23-24.4 For FcyRI, three different genes (FcyRIa, FcyRIb, and FcyRIC) exist that encode four distinct transcription products. From the prediction of molecular analysis, only FcyRIa and FcyRIb give rise to transmembrane receptors, whereas FcyRIb2 and FcyRIC presumably encode soluble products.4 Expression of FcyRI was found to be restricted mainly to mononuclear phagocytes, but polymorphonuclear phagocytes (PMN) can express FcyRI in certain clinical situations5,7 and after in vitro incubation with interferon-γ (IFN-γ).8 We recently described FcyRII expression on PMN of patients receiving granulocyte colony-stimulating factor (G-CSF) after chemotherapy for head and neck cancer.9 We show here that expression of FcyRI is a characteristic feature of PMN during therapy with this cytokine.

Decreased granulocyte counts (due to chemotherapy or to primary hematologic disorders) are associated with an increased risk of bacterial or fungal infections.10 Therefore, clinical application of hematopoietic growth factors, which regulate the proliferation and differentiation as well as the functional activity of myeloid cells, is under intensive investigation. Among this expanding family of glycoproteins, G-CSF has shown its capability to ameliorate neutropenia and its sequelae.11,12 Although in vitro incubation of PMN with G-CSF does enhance the functional activity of PMN in different assays,13 no major change in Fc receptor expression, especially no significant induction of FcyRI, could be observed after in vitro incubation.4 After in vitro induction by IFN-γ, FcyRI on PMN mediates phagocytosis, generation of the oxidative burst, and tumor cell cytotoxicity,2 supporting a role as an important trigger molecule on PMN.

Therapeutically, a combination of G-CSF and antibodies may be promising for the management of certain infections. In addition, expression of FcyRI may increase the interest in neutrophils as putative effector cells in a concept of targeted cytotoxicity.14 Neutrophils can lyse a variety of different tumor cell lines, but also tumor cells obtained from patients.15 As shown here, PMN-mediated tumor cell cytotoxicity is enhanced during G-CSF application directly involving FcyRI as a cytotoxic trigger molecule.

MATERIALS AND METHODS

Monoclonal Antibodies (MoAbs)

Irrelevant antibody (IgG2a), used as a control in immunofluorescence analyses, was produced from the hybridoma clone 10.3-6.2.

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Bispecific Antibody 520C9 x 22 [F(ab')2 x F(ab')2]

Bispecific antibody 520C9 x 22 [F(ab')2 x F(ab')2] was prepared by Dr Kwan-Sa You (Medarex). Briefly, F(ab')2 fragments of MoAb 520C9 (IgG1)18 to the erbB2/HER-2 proto-oncogene product and antibody AT1022 to FcyRII were kindly provided by Dr M.J. Glenie (Tenovus Research Laboratory, Southampton, UK). MoAb 425 (mouse IgG2a)19 to the epidermal growth factor (EGF) receptor was provided by Dr G. A. Luckenbach (Merck, Darmstadt, Germany). Fluorescein isothiocyanate (FITC)-conjugated F(ab'), fragments of antihuman IgG antibodies detecting IgA, IgM, and IgG were used as the secondary antibody for indirect immunofluorescence. FITC-conjugated F(ab') fragments of antihuman IgG antiserum were from Cappel (Cochrane, PA).

Target Cell Lines

A 1207 (glioblastoma), originally established by Dr S.A. Aaronson (Laboratory of Cell and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda), and A431 (squamous cell carcinoma)20 were kindly provided by Merck and cultured in Dulbecco's medium (GIBCO, Paisley, UK) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO), 4 mmol/L L-glutamine (GIBCO), and 10% fetal calf serum (PCS; GIBCO). Sk-ov3 (ovarian carcinoma) obtained from ATCC were grown in RPMI 1640 (Seromed, Berlin, Germany) supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin (GIBCO), 4 mmol/L L-glutamine (GIBCO), and 10% fetal calf serum (PCS; GIBCO). Neutrophil Isolation

PMN were isolated by a modified method slightly modified from that described in Repp et al.9 Briefly, heparin-anticoagulated blood was layered over a discontinuous Percoll gradient (Seromed) consisting of two layers of 68% and 63% Percoll, respectively. After centrifugation, PMN were harvested from the Percoll interface and the remaining erythrocytes were removed by hypotonic lysis. Cytospin preparations were used to assess the purity of isolated cells, which was higher than 95%, with few contaminating eosinophils. The viability of cells, determined by trypan blue exclusion, exceeded 95%.

Table 1. Expression of FcyRI During G-CSF Therapy

<table>
<thead>
<tr>
<th>Disease</th>
<th>Median rFI</th>
<th>Range</th>
<th>n</th>
</tr>
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<tbody>
<tr>
<td>Solid tumors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head and neck cancer</td>
<td>4.5</td>
<td>2.1-8.3t</td>
<td>6</td>
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<tr>
<td>Cancer of the testis</td>
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<td>1</td>
</tr>
<tr>
<td>Lymphoma</td>
<td></td>
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</tr>
<tr>
<td>Hodgkin's disease</td>
<td>2.4</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>High-grade NHL</td>
<td>2.5</td>
<td>1.7-10.3*</td>
<td>3</td>
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<tr>
<td>Leukemia</td>
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<tr>
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<td>1</td>
</tr>
<tr>
<td>Acute lymphoblastic leukemia</td>
<td>7.9</td>
<td>6.8-8.2t</td>
<td>3</td>
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<tr>
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<td></td>
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<tr>
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<td>1</td>
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<td>1</td>
</tr>
<tr>
<td>Cyclic neutropenia</td>
<td>10.7</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Chronic granulomatous disease</td>
<td>2.7</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>All G-CSF-treated patients</td>
<td>5.3</td>
<td>1.7-10.3†</td>
<td>19</td>
</tr>
<tr>
<td>Healthy donors</td>
<td>1.0</td>
<td>1.0-1.1</td>
<td>8</td>
</tr>
</tbody>
</table>

rFI for FcyRI was analyzed by indirect immunofluorescence after staining with MoAb 32.2. PMN from patients with different diseases receiving G-CSF were compared with PMN from healthy donors. Data from n numbers of different donors are presented as median and range of rFI as indicated.

* P < .05.
† P < .01.
of goat antimouse MoAb were added for 30 minutes at 4°C. Cells were washed again and resuspended in PBS for analysis on a flow cytometer (EPICS Profile; Coulter, Hialeah, FL). To compare results, relative fluorescence intensities (rFI) were calculated as the ratios of linear fluorescence intensities (FI) obtained with relevant (rel) relative to irrelevant (irrel) antibodies (FI rel MoAb/FI irrel MoAb).

**Northern Analysis of RNA**

Total cellular RNA was isolated from various cells using the RNA zol B method (Cinna Biotec, Friendswood, TX) as in Kindt et al.26 Cell lines used were K562 (FcyRI negative control), U937, and THP-1 (both FcyRI positive), U937 and THP-1 were cultivated for 24 hours with 100 U/mL IFN-γ before RNA isolation. PMN from different patients were isolated as above. Upon isolation, 10 μg of total cellular RNA was fractionated by electrophoresis in 1% agarose/formaldehyde gels and transferred to nitrocellulose (BA85; Schleicher & Schuell, Dassel, Germany). Hybridization with 32P-labeled 1.4-kb Xho fragment of cDNA p13528 was performed overnight in 50% formamide, 5× SSC, 0.1% sodium dodecyl sulfate (SDS), 10× Denhardt’s solution, with 50 μg/mL denatured Salmon sperm DNA at 42°C. Filters were washed in 0.3× SSC, 0.1% SDS at 42°C for 30 minutes and exposed to Kodak XAR5 (Eastman Kodak, Rochester, NY).

**Identification of Different Gene Products by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)**

Five micrograms of total RNA were reverse transcribed using 17 U of Avian Moloney virus reverse transcriptase (PharMacia; Uppsala, Sweden) for 90 minutes at 42°C. Incubations were performed in 50 μL with 10 mmol/L MgCl₂, 100 mmol/L Tris-HCl (pH 8.3), 140 mmol/L KCl, 2 mmol/L dithiothreitol (DTT), 0.2 mmol/L of each dNTP, and 100 ng of Not I-d(T)₄ primer (PharMacia). Three microliters of RT-cDNA was added to a PCR reaction (100 μL volume) with 1× PCR buffer (Promega, Madison, WI), 0.25 mmol/L of each dNTP, 10 pmol of each oligonucleotide primer, and 1 U Taq polymerase (Perkin-Elmer, Norwalk, CT). FcyRI transcripts were amplified using primer 9 (5'-ACACCCACAAAAGGCTGTA-3'), corresponding to nt 89-106, and primer 10 (5'-CACCCAGAG-TACCTGTTT-3'), corresponding to the reverse complement of nt 952-969 of cDNA p135.5 PCR amplification was performed in an automated thermal cycler (Bio Phase; Bio Excellence, Colchester, UK) using denaturation for 2 minutes, primer annealing at 55°C, and extension at 72°C for 2 minutes (30 cycles). Twenty microliters of PCR products was fractionated by agarose gel electrophoresis and blotted to nitrocellulose. Specific FcγRIa and Ib products were detected using gene-specific oligonucleotide probes 37A (5'-GAATATCTGTCACTGTGA-3') and 39B (5'-GAATATCGATTACATCACTG-3'). Oligonucleotides were 5'-phosphorylated with 32p and polynucleotide kinase (PharMacia).

Neutrophil RT-cDNA samples were checked for potential monocYTE contamination by amplification of the c-fms CDNA (encoding the macrophage colony-stimulating factor [M-CSF] receptor), which is expressed in monocyes, but not in PMN.26 We used an exon 19 sense primer (5'-TCATTTGCGCTGATCCTCTA-3') and an exon 22 antisense primer (5'-TCCCTGTTCGCTCAACTTGCTCA-3'). PCR amplification was performed exactly as in Kaashoek et al.26 and never yielded the characteristic 389-bp c-fms band in any RT-cDNA sample derived from RNA isolated from PMN with G-CSF patients.

**Binding of Human IgG**

Neutrophils were isolated as above and incubated in RPMI for 3 hours (37°C; 5% CO₂) and cells were washed twice with RPMI 1640. Cells were divided into two fractions, one was incubated (30 minutes at 4°C) in RPMI 1640 alone and the other was incubated with RPMI 1640 and monomeric polyclonal human IgG (20 μg/mL; ultracentrifuged at 100,000g for 1 hour). After washing once, cells were stained with FITC-conjugated F(ab) fragments specific for human IgG. Fluorescence intensity was quantitated by flow cytometry.

**Antibody-Dependent Cellular Cytotoxicity (ADCC) Assays**

Cytotoxicity assays were performed as described.20 Briefly, target cells (A1207, A431, or Sk-ov3) were labeled with 3Cr (100 μCi for 3 hours) and sensitized by saturating concentrations of MoAbs as determined by immunofluorescence. MoAb 425 (20 μg/mL) to the EGF receptor was used for A1207 and A431. For Sk-ov3, either bispecific F(ab')₂ X F(ab')₂ antibody 520C9 x 22 (20 μg/mL) or an equimolar mixture of unlinked F(ab')₂ fragments of both parental antibodies was used. After incubation of targets (37°C; 5% CO₂ for 30 minutes), cells were washed once and numbers were adjusted to give the desired amount of target cells in 50 μL of RF10+. Neutrophils (50 μL), target cells (50 μL), and RF10+ were added to a final volume of 200 μL. When indicated, conditioned medium of bladder carcinoma cell line 5637 (50 μL) was added instead of RF10+ during ADCC assays. This conditioned medium is a source of hematoxigous growth factors and was used to accomplish in vitro stimulation of effector cells. In control experiments, it was found to not be cytotoxic to target cells and to not alter FcγRI expression on PMN. In some experiments, anti-Fcγ receptor antibodies (10 μg/mL final concentration) were added 30 minutes before the addition of targets. Effector to target ratios were 40:1 for A1207 and A431 and 80:1 for Sk-ov3, which were determined in pilot experiments to give comparable amounts of tumor cell lysis. Assay duration was 3 hours for A1207 and A431 and 18 hours for Sk-ov3, starting at the addition of target cells.

The release of label from triplicates was measured and analysed using the formula:

\[
\text{Specific Lysis} = \frac{\text{Experimental cpm} - \text{Spontaneous cpm}}{\text{Maximum cpm} - \text{Spontaneous cpm}} \times 100
\]

For analysis of effects induced by anti-Fc receptor antibodies, % inhibition was calculated:

\[
\% \text{ Inhibition} = \frac{- \text{Lysis in the Presence of Antibody}}{- \text{Lysis in Control Medium}} \times 100
\]

Negative values determined by this formula are reported as % stimulation in the presence of antibody (only observed in case of MoAb 197 and PMN from healthy donors).

**Statistical Analysis**

Statistical differences were analyzed by unpaired Student’s t-test or, when appropriate, paired Student’s t-test. Significance was accepted when P < .05. Group data are reported as the mean ± standard error of the mean (SEM), or as the median and range of the number (n) of experiments, as indicated.

**RESULTS**

**Presence of FcγRI on Neutrophils**

**FcγRI expression during G-CSF therapy.** Expression of the Fc receptor with high affinity for IgG (FcγRI; CD164) was analyzed on isolated PMN by indirect immunofluorescence staining with MoAb 32.2. Thus, neutrophils of patients receiving G-CSF (3 to 5 μg/kg body weight) expressed
significantly ($P < .01$) more FcγRI (median rFI, 5.3; range, 1.7 to 10.3; $n = 19$) than did PMN from healthy donors (median rFI, 1.0; range, 1.0 to 1.1; $n = 8$). In this study, PMN from patients with various malignancies and different hematologic disorders were analyzed during G-CSF therapy (Table 1). So far, all tested patients showed marked expression of FcγRI during G-CSF treatment. PMN from individual patients with different diseases (head and neck cancer, non-Hodgkin's lymphoma [NHL], and cancer of testis) were analyzed before and during G-CSF application. The results of staining on PMN from these patients for FcγRI (rFI without/with G-CSF) were 1.4/8.4, 0.9/2.3, and 1.5/3.4, respectively. Neutrophils from one patient with cyclic neutropenia markedly expressed FcγRI during G-CSF (rFI = 10.7) but not during GM-CSF (rFI = 0.9) treatment. These data support the expression of the high-affinity receptor for IgG to be a characteristic feature of PMN during G-CSF therapy.

Fc receptors are members of the Ig-supergene family consisting of a large number of highly homologous molecules. To test whether binding of MoAb 32.2 to PMN during rhG-CSF therapy was due to cross-reactivity with other members of this family, a panel of six CD64 antibodies was evaluated. Figure 1 shows the results of an experiment comparing PMN from a healthy donor and a patient with high-grade lymphoma receiving G-CSF. Significant binding of anti-CD64-specific antibodies occurred only with PMN during G-CSF treatment. Differences in staining intensities between the various antibodies are in agreement with known differences in binding affinity to FcγRI. Similar results were obtained with PMN from a patient treated with G-CSF for aplastic anemia (data not shown). As these antibodies recognize at least three different epitopes of FcγRI, these results further support the idea that PMN during G-CSF treatment indeed express the high-affinity receptor for IgG.

Northern analysis of FcγRI. We next analyzed whether FcγRI was actively transcribed in PMN upon exposure to G-CSF in vivo. As a control, we used the myeloid THP-1 cell line, which showed the characteristic 1.7- and 1.6-kb transcripts for FcγRI. The 1.6-kb FcγRI transcript was only faintly visible in freshly isolated monocytes or monocytic cell line THP-1 (data not shown), but expression increased dramatically upon overnight culture with IFN-γ. These FcγRI transcripts were absent in K562. RNA isolated from PMN from four patients treated with G-CSF expressed FcγRI mRNA of the 1.7-kb size (Fig 2). PMN from patient no. 5 were evaluated both before and during treatment with G-CSF, and only showed the 1.7-kb FcγRI transcript during the treatment episode. These data support the idea that FcγRI is actively transcribed in PMN upon treatment of patients with G-CSF in vivo.

Detection of different FcγRI transcripts. Three highly homologous genes have been found to encode four distinct FcγRI transcripts. Due to this extensive homology between the three genes (>98% sequence identity), the different transcripts can only be selectively detected via RT-PCR analysis. *RT-cDNA products from three patients were used to amplify FcγRI transcripts via primers hybridizing to the first extracellular Ig-like domain (EC1) encoding region and the cytoplasmic tail encoding domain. Upon amplification two PCR products were consistently observed. The blotted PCR products were probed with different gene-specific primers. The largest (880 bp) band hybridized exclusively with an FcγRIIA gene-specific primer (Fig 3A) and corre-
sponds to a transcript encoding an \( \text{Fc}\gamma R I \) molecule containing three Ig-like extracellular domains.\(^4\) Interestingly, the smaller (600 bp) product hybridized selectively with an \( \text{Fc}\gamma R I B/C \)-specific primer (Fig 3B) and was recently found to constitute solely of an \( \text{Fc}\gamma R I \)-encoded transcript. This transcript (\( \text{Fc}\gamma R I b2 \)) lacks one of the extracellular domains (ie, EC3). The simultaneous expression of both these \( \text{Fc}\gamma R I a \) and \( \text{Fc}\gamma R I b2 \) products parallels our observations with other \( \text{Fc}\gamma R I \)-positive cells analyzed up to now, including monocytes,\(^4\) U937 (Fig 3), and THP-1 cells (data not shown).

All PMN samples from patients analyzed in this study consisted of more than 95% of neutrophils, as evaluated morphologically (containing cells being mainly eosinophils). To exclude the possibility of monocyte contamination, causing (false-) positive PCR results, we subjected all PMN RT-cDNA samples (Fig 3) to a PCR method to detect the monocyte-specific \( c\text{-}fms \) transcript.\(^28\) However, in none of our PMN samples could we identify the \( c\text{-}fms \) cDNA product consistent with absent monocytes.

**Functional activity of neutrophil F\( \gamma R I \)**

**Binding of monomeric human IgG.** F\( \gamma R I \) differs from both classes of low-affinity IgG receptors, F\( \gamma R II I \) (CD32) and F\( \gamma R III I \) (CD16), by virtue of its capacity to bind monomeric IgG with high affinity. Initially, in vivo-bound cytophilic IgG was analyzed after 3 hours of incubation of PMN in serum-free medium. After washing, bound IgG was detected using FITC-conjugated F(ab’) fragments for human IgG (Fig 4). In these experiments, more surface IgG was found on PMN during G-CSF therapy compared with that found on control PMN (mean FI, 23.9 ± 7.8 SEM vs 6.9 ± 2.9; \( n = 3 \)). Subsequently, monomeric human IgG was added, cells were incubated for another 30 minutes at 4°C, and cells were analyzed as above (Fig 4). Thus, minimal binding of exogenous IgG was observed with control PMN (FI increased from 6.9 ± 2.9 to 8.4 ± 3.3, \( n = 3 \)). In contrast, G-CSF PMN bound increased amounts of monomeric IgG (FI, 23.9 ± 7.8 to 45.3 ± 20.8; \( n = 3 \)). These data show that G-CSF PMN were capable of binding significantly (\( P < .05 \)) more human IgG than control PMN.

**ADCC.** Enhanced cytotoxicity of PMN during in vivo application of G-CSF was observed using Daudi lymphoma cells as targets after sensitization with polyclonal rabbit antiserum.\(^9\) Further experiments were performed to investigate whether these results can be extended to solid tumor cell targets, using MoAbs for sensitization. Therefore, ADCC assays of PMN against A1207 (glioma) and A431 (squamous cell carcinoma) cell lines were established after the sensitization of target cells with MoAb 425 to the EGF receptor. As shown in Table 2, neutrophils during G-CSF therapy were significantly (\( P < .001 \) and \( P < .05 \)) more cytotoxic to A1207 and to A431, respectively, than neutrophils from healthy donors.

**Identification of Fc receptors involved in ADCC.** F\( \gamma R I \) has been suggested to be the main trigger molecule for cytotoxicity of freshly isolated PMN, but (after in vitro induction by IFN-\( \gamma \) ) F\( \gamma R I \) was also shown to mediate killing of tumor cells.\(^3\) Accordingly, expression of F\( \gamma R I \) during G-CSF treatment correlated with enhanced cytotoxicity to Daudi lymphoma cells.\(^9\) To show involvement of F\( \gamma R I \) more directly, inhibition experiments with blocking antibodies to F\( \gamma R I \) (MoAb 197; 10 \( \mu g/\text{mL} \); whole antibody), F\( \gamma R II I \) [MoAb IV.3; 10 \( \mu g/\text{mL} \); F(ab’)] fragments, or both (10 \( \mu g/\text{mL} \) each) were performed. MoAb 197 binds outside...
the ligand binding site of FcγRII and blocks IgG binding to FcγRI via its IgG2a Fc fragment ("Kurlander effect").

The selectivity of this inhibition for FcγRI has been shown before. For these blocking experiments, PMN from healthy donors were stimulated in vitro by adding conditioned medium of bladder carcinoma cell line 5637 during ADCC assays, because cytotoxicity of control PMN was too low to investigate inhibitory effects (Table 2). This conditioned medium (containing natural human GM-CSF [nGM-CSF] and nhG-CSF) did not alter FcγRI expression on PMN. A1207 cells opsonized by MoAb 425 (IgG2a) were used as targets. Thus, cytotoxicity of PMN from healthy donors was not inhibited by MoAb 197 to FcγRI (mean of % specific lysis 26.2 ± 14.8 SEM without MoAb 197 v 27.8 ± 16.8 with MoAb 197), whereas significant (P < .05) inhibition of PMN from G-CSF–treated patients occurred (33.3 ± 17.9 without v 15.1 ± 18.4 with MoAb 197). MoAb IV.3 to FcγRII, on the other hand, showed significant (P < .01) inhibition only on neutrophils from healthy donors (26.2 ± 14.8 v 3.7 ± 4.2), but did not affect cytotoxicity of G-CSF PMN (33.3 ± 17.9 v 29.6 ± 17.3). Using a combination of both antibodies, cytotoxicity of PMN from healthy donors (26.2 ± 14.8 v 2.4 ± 4.2) and G-CSF–treated patients (33.3 ± 17.9 v 5.5 ± 8.6) was found to be significantly (P < .01 and P < .05, respectively) inhibited. To show the different effects of these anti-Fc receptor antibodies on both PMN populations (either from healthy donors or from G-CSF patients) more clearly, % inhibition (see Materials and Methods) was calculated from the data reported above and displayed in Fig 5. Similar results were obtained in a control experiment using F(ab') fragments of MoAb AT10 instead of F(ab) fragments of MoAb IV.3 against FcγRII (92.5% inhibition of control PMN v 1.4% stimulation of G-CSF PMN). Thus, PMN from healthy donors and from G-CSF–treated patients differed substantially in their susceptibility to inhibition by anti-Fc receptor antibodies.

Selective ADCC via FcγRI. These blocking studies further supported cytotoxicity by PMN from G-CSF–treated patients to be mediated via FcγRI. To assess the functional role of FcγRI more directly, bispecific antibody 520C9,9 with specificities against both the tumor–associated antigen erbB2/HER-2 and FcγRI was used. Ovarian carcinoma cells (SK-ov-3), overexpressing erbB2/HER-2, were used as targets. Assay duration was 18 hours in the presence of conditioned medium of 5637 cells for in vitro stimulation of effector cells. Control experiments with an equimolar mixture of unlinked F(ab') fragments of both parental antibodies gave only marginal levels of target killing (less than 20% of bispecific lysis). Neutrophils during G-CSF therapy were found to be significantly (P < .05) more efficient in killing via FcγRI than PMN from healthy donors (mean of % specific lysis, 20.1 ± 12.1 SEM, [n = 6] v 4.1 ± 1.7 [n = 6], respectively) (Fig 6). These data indicate that FcγRI on PMN may act as cytotoxic trigger molecule in a concept of targeted cytotoxicity with bispecific antibodies.

**DISCUSSION**

Of all three classes of Fc receptors for IgG, the high-affinity receptor (FcγRI; CD64) is the one with the most restricted cellular distribution. It is constitutively expressed only on peripheral blood monocytes, on macrophages, and (albeit at very low numbers) on PMN.2 Our previous observation9 that neutrophils during G-CSF therapy express large numbers of FcγRI is now confirmed in patients with a broad spectrum of hematologic and nonhematologic disorders. Originally, patients receiving chemotherapy were randomized to either subcutaneous G-CSF injections or to a control arm treated with the same chemotherapy but without G-CSF support.9 FcγRI expression was found to be sig-

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**Table 2. Cytotoxicity of PMN During G-CSF Treatment**

<table>
<thead>
<tr>
<th></th>
<th>A1207 Healthy Donors</th>
<th>A1207 G-CSF Patients</th>
<th>A431 Healthy Donors</th>
<th>A431 G-CSF Patients</th>
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<tr>
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<td>SEM</td>
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</table>

ADCC of PMN from G-CSF–treated patients and from healthy donors was compared using MoAb 425 (IgG2a) to the EGF receptor for the sensitization of A1207 (glioma) and A431 (squamous cell carcinoma) cell lines. The differences between PMN from G-CSF–treated patients and from healthy donors were significant (P < .001 for A1207; P < .05 for A431) for both cell lines.
significantly higher on PMN from G-CSF-treated patients compared with control patients. In this study, individual patients with different diseases were investigated for FcγRI expression before and during G-CSF treatment. On PMN from these patients, markedly higher FcγRI expression was found during G-CSF treatment. Together, these data suggest expression of FcγRI to be triggered by exogenous G-CSF and not the chemotherapy regimen or patients' disease, although these latter factors may also contribute to FcγRI expression in individual patients. As FcγRI belongs to the Ig-supergene family of highly homologous molecules, a panel of six CD64 antibodies was tested to exclude cross-reactivity. In addition, mRNA for FcγRI was shown in PMN isolated from patients during G-CSF therapy. These data show FcγRI expression on PMN as a general phenomenon during G-CSF therapy.

At the moment, the mechanism of FcγRI expression on PMN during G-CSF application is incompletely understood. IFN-γ, but not G-CSF, can induce FcγRI on PMN during in vitro culture. On peripheral blood PMN, increased expression of FcγRI was shown upon treatment with IFN-γ and in certain clinical situations. Accordingly, expression of FcγRI on peripheral blood PMN has been suggested as a possible indicator for the presence of IFN-γ in vivo. Our results indicate that endogenous production of G-CSF also needs to be considered as an explanation for FcγRI expression in these situations. Indeed, increased levels of endogenous G-CSF have been reported accompanying bacterial infections as well as recovery from myeloablative chemotherapy. Clinical situations in which enhanced FcγRI expression on PMN can be observed. Neutrophil precursors in the bone marrow are known to express FcγRI, and G-CSF reduces the maturation time of PMN precursors in the bone marrow. Therefore, increased FcγRI expression of PMN during G-CSF therapy may be due to its action on neutrophil progenitors in the bone marrow. Accordingly, during treatment with GM-CSF, which does not reduce the maturation time of PMN in the bone marrow, FcγRI expression was found not to be enhanced in a patient with cyclic neutropenia. Recently, G-CSF has been shown to induce FcγRI expression on highly enriched CD34+ bone marrow cells.

So far, three genes (termed FcγRIA, FcγRIB, and FcγRIC) have been identified that encode four transcripts for the high-affinity receptor for IgG (FcγRI; CD64). RT-PCR analysis was performed using FcγRIa- and FcγRIb/c-specific primers, respectively, to see whether also FcγRIB and FcγRIC transcripts were present in neutrophils during G-CSF therapy. These experiments clearly showed transcription of both the membrane bound isoforms, FcγRIa and FcγRIB, and FcγRIC, which was stronger in neutrophils. Therefore, all three domains seem necessary for high-affinity binding. Up to now, the biologic role of the "two-domain" hFcγRIB2 molecule, as well as of the two soluble forms (hFcγRIB1 and hFcγRIC), is obscure and is the subject of detailed analysis.

On myeloid cells, FcγR mediate diverse functions such as phagocytosis; the triggering of cytotoxicity; degranulation; the generation of superoxide; the release of cytokines such
as interleukin-1 (IL-1), IL-6, and tumor necrosis factor-α (reviewed in Van de Winkel and Anderson); and the enhancement of antigen presentation, suggesting an important role as biologic trigger molecules. However, the precise classes or isoforms of FcγR mediating these various effects on different populations of effector cells remain to be identified for most functions. Neutrophil-mediated cytotoxicity of tumor cells was shown to be triggered via FcγRII, using either bispecific antibodies (with tumor- and FcR-directed specificities), or anti-FcR antibody-producing hybridomas as targets in reverse ADCC. In these assays, which most selectively engage distinct classes of FcγR, FcγRI, after in vitro induction by IFN-γ, also mediate tumor cell cytotoxicity by neutrophils. Our data presented here, using bispecific antibodies and blocking antibodies to the different classes of FcγR, establish an active role of FcγRI on PMN in vivo.

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Involvement of the high-affinity receptor for IgG (Fc gamma RI; CD64) in enhanced tumor cell cytotoxicity of neutrophils during granulocyte colony-stimulating factor therapy

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