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

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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 transcription 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 F(ab')2 X F(ab')2-bispecific 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 1q23-24. 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 FcyRIB2 give rise to transmembrane receptors, whereas FcyRIB1 and FcyRIC presumably encode soluble products. Expression of FcyRI was found to be restricted mainly to mononuclear phagocytes, but polymorphonuclear phagocytes (PMN) can express FcyRI in certain clinical situations and after in vitro incubation with interferon-γ (IFN-γ). We recently described FcyRI expression on PMN of patients receiving granulocyte colony-stimulating factor (G-CSF) after chemotherapy for head and neck cancer. 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. 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. Although in vitro incubation of PMN with G-CSF does enhance the functional activity of PMN in different assays, no major change in Fc receptor expression, especially no significant induction of FcyRI, could be observed after in vitro incubation. After in vitro induction by IFN-γ, FcyRI on PMN mediates phagocytosis, generation of the oxidative burst, and tumor cell cytotoxicity, 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. Neutrophils can lyse a variety of different tumor cell lines, but also tumor cells obtained from patients. 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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Submitted November 30, 1992; accepted March 19, 1993.

Supported in part by the Deutsche Forschungsgemeinschaft (Grant No. GR 689/3-1).

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Blood, Vol 82, No 3 (August 1), 1993: pp 931-939
 obtained from American Type Culture Collection (ATCC; Rockville, MD). Whole antibodies to FcγRI (32.2, IgG1^1); 22, IgG1^17; 197, IgG2a^17), FcγRII (IV.3, IgG2b^19), and FcγRIII (3G8, IgG1^19); Fab fragments of IV.3; and F(ab')2 fragments of 3G8 were obtained from Medarex (West Lebanon, NH). Anti-FcγRI antibodies 44 (IgG1)^17 and 62 (IgG1)^17 were kindly provided by Dr. P.M. Guyre (Dartmouth Medical School, Lebanon, NH); whole antibody 10.1 (IgG1)^19 was a kind gift from Dr. N. Hogg (Imperial Cancer Research Fund, London, UK); whole antibody 197 was shown to inhibit FcγRI-mediated binding selectively.21 F(ab')2 fragments of antibody AT10^22 to FcγRII were kindly donated by Dr. M.J. Glennie (Tenovus Research Laboratory, Southampton, UK). MoAb 425 (mouse IgG2a)^23 to the epidermal growth factor (EGF) receptor was provided by Dr. G.A. Lunkencb (Merck, Darmstadt, Germany). Fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragments of goat antimouse antibody detecting IgA, IgM, and IgG were used as the secondary antibody for indirect immunofluorescence. FITC-conjugated F(ab')2 fragments of antihuman IgG antiserum were from Cappel (Cocranville, PA).

**Bispecific Antibody 520C9 × 22 (F(ab')2 × F(ab')2)**

Bispecific antibody 520C9 × 22 (F(ab')2 × 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 of MoAb 22 (IgG1) to FcγRII^17 were prepared by peptide digestion of their respective parent antibodies. Conjugation was obtained via thioether linkage between an artificially introduced sulfhydryl group (from SATA) on antibody 520C9 and a maleimido group (from SMCC) on antibody 22.

SATA derivatization of F(ab')2 fragments of MoAb 520C9 was performed by incubating 0.2 μmol (30 mg) of MoAb 520C9 in 5 mL of sodium phosphate-2.5 mmol/L EDTA (buffer), pH 7.4, with 10 times molar excess of SATA (Pierce, Rockford, IL). The reaction was stopped by adding 1 mol/L glycine solution to a final concentration of 0.01 mol/L. Excess SATA and glycine were removed by dialysis against buffer. To expose the -SH group, SATA-derivatized F(ab')2 fragments were treated with 50 mmol/L hydroxyamine (1 hour at 21°C), followed by dialysis. Sulfo-SMCC derivatization of F(ab')2 fragments of MoAb 22 was obtained by incubating 0.2 μmol (30 mg) of MoAb 22 in 5 mL buffer with 20 times molar excess of Sulfo-SMCC (Pierce). The reaction was terminated as described above. Thioether linkage was formed by incubating SATA-derivatized, −SH exposed F(ab')2 fragments of MoAb 520C9 and SMCC-derivatized F(ab')2 fragments of MoAb 22 at 1:1 molar ratio (17 hours at 21°C). The reaction was stopped by adding solid NEM to a final concentration of 10 mmol/L for 1 hour. Excess NEM was removed by dialysis.

**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)^25 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 as Dulbecco’s medium (RF10^29), including 0.07 U/mL human insulin ( Hoechst, Frankfurt, Germany).

**Patients**

Patients with advanced (stage III or IV) squamous cell carcinoma of the oral cavity and pharynx (head and neck cancer) were treated according to the protocol of the clinical trial GCSF8806. Details of this trial (which was approved by the Institutional Review Board of the Medical Faculty, University of Erlangen-Nürnberg, in accordance with the Declaration of Helsinki) were reported in Repp et al.9 Since the approval of recombinant human G-CSF (rhG-CSF) by the German Drug Administration, patients with various disorders and different therapies (Table 1) were treated with rhG-CSF (Neupogen, 3 to 5 μg/kg of body weight) based on clinical indications. In these patients, the kinetics of neutrophil recovery during G-CSF treatment were variable. Therefore, the criteria used to analyze PMN from these patients were defined as (1) at least 3 days of G-CSF treatment and (2) absolute neutrophil counts higher than 2,500/μL. PMN from one patient with cyclic neutropenia were analyzed during either rhG-CSF or recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) (3.5 μg/kg of body weight; Behring, Marburg, Germany) treatment using the same criteria as above.

**Neutrophil Isolation**

PMN were isolated by a 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. Cytosin 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%.

**Receptor Analysis**

Cells were isolated as described above and were stained with MoAb. During the antibody incubation, polyclonal human IgG (4 mg/mL) was added to inhibit nonspecific binding to FcγRI. After washing the cells four times in phosphate-buffered saline (PBS) + 1% bovine serum albumin (BSA), FITC-labeled F(ab')2 fragments of...
were washed again and resuspended in PBS for analysis on a flow cytometer (EPICS Profile; Coulter, Hialeah, FL). To compare ratios, 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 Biotex, Friendswood, TX) as in Kindt et al.28 Cell lines used were K562 (FcyRI negative control), U937, and THP-1 (both FcyRI positive), U937 and THP-1 were cultured 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 p13557 was performed overnight in 50% formamide, 3x SSC, 0.1% sodium dodecyl sulfate (SDS), 10x Denhard’s solution, with 50 µg/mL denatured Salmon sperm DNA at 42°C. Filters were washed in 0.3x 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)18 primer (Pharmacia). Three microliters of RT-cDNA was added to a PCR reaction (100 µL volume) with 1x 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 (S-ACACCACAAAGGCAGTGAACTTCTGCACTGTCAACTCTGTTTGGTCCTCCGACCATGGTGA-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 hFcyRIa and Ib products were visualized using autoradiography and quantitated using a densitometer (Ultrascan XL; Pharmacia).

Neutrophil RT-cDNA samples were checked for potential monocytogenes 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'-TCATTTGCGTCAATCCCTA-3') and an exon 22 antisense primer (5'-TCCCTGTGCTCAACTCTTGTGCTGA-3'). PCR amplification was performed exactly as in Kaasheko et al26 and never yielded the characteristic 389-bp c-fms band in any RT-cDNA sample derived from RNA isolated from PMN or 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)² fragments specific for human IgG. Fluorescence intensity was quantitated by flow cytometry.

Antibody-Dependent Cellular Cytotoxicity (ADCC) Assays
Cytotoxicity assays were performed as described.29 Briefly, target cells (A1207, A431, or Sk-ov3) were labeled with 51Cr (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×F(ab)² antibody 520C9×22 (20 µg/mL) or an equimolar mixture of unlabeled 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 hematopoietic growth factors29 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 analyzed 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:

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

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; CD64) 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 FcγRI A gene-specific primer (Fig 3A) and corre-
treated with G-CSF. PMN from one patient (P5) were analyzed before and during G-CSF therapy (as indicated). The positions of 18S and 28S ribosomal RNAs are marked.  

Fig 2. Northern analysis of hFcγRI transcripts in PMN from G-CSF–treated individuals. Total cellular RNA was isolated from Fcγ-RII–expressing THP-1 cells (cultured for 24 hours with IFN-γ), K562 cells (FcγRII negative), and PMN isolated from four patients treated with G-CSF. PMN from one patient (P5) were analyzed both before and during G-CSF therapy (as indicated). The positions of 28S and 18S ribosomal RNAs are marked.

Fig 3. Expression of hFcγRI transcripts in PMN from patients treated with G-CSF analyzed via RT-PCR. Total cellular RNA was isolated from Fcγ-RII–expressing U937 cells (cultured for 24 hours with IFN-γ) and from PMN of three G-CSF–treated patients. RNA was reverse-transcribed and PCR amplified using FcγRI-specific primers. This procedure was followed by electrophoretic separation, blotting to nitrocellulose, and hybridization with end-labeled oligonucleotides. (A) hFcγRIa transcripts (present in 880-bp PCR band) were detected by an hFcγRIa-specific primer, and (B) hFcγRIIb2 (present in 600-bp band) via an hFcγRIIIb-specific primer, as described in Material and Methods. Hybridizing fragments are shown with sizes marked (bp).

Functional activity of neutrophil FcγRI

Binding of monomeric human IgG. FcγRI differs from both classes of low-affinity IgG receptors, FcγRII (CD32) and FcγRIII (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 v 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. 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 polyclonal rabbit anti-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. FcγRII has been suggested to be the main trigger molecule for cytotoxicity of freshly isolated PMN, but (after in vitro induction by IFN-γ) FcγRI was also shown to mediate killing of tumor cells. Accordingly, expression of FcγRI during G-CSF treatment correlated with enhanced cytotoxicity to Daudi lymphoma cells. To show involvement of FcγRII more directly, inhibition experiments with blocking antibodies to FcγRII (MoAb 197; 10 μg/mL; whole antibody), FcγRII [MoAb IV.3; 10 μg/mL; F(ab') fragments], or both (10 μg/mL each) were performed. MoAb 197 binds outside...
the ligand binding site of FcγRII and blocks IgG binding to FcγRI through 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 [nhGM-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–treated 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 ([F(ab'),] with specificities against both the tumor-associated antigen erbB2/HER-2 and FcγRI was used. Ovarian carcinoma cells (SK-ov3), 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. Our previous observation 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. FcγRI expression was found to be sig-
FCγRI ON PMN

Fig 5. Role of FCγRI and FCγRII in the cytotoxicity of PMN isolated from healthy donors or patients during G-CSF therapy (G-CSF). Saturating amounts of blocking antibodies to FCγRI (MoAb 197: whole antibody), to FCγRII (MoAb IV.3: Fab fragments), or to both were added to ADCC assays against A1207 tumor cells that were sensitized with MoAb 425 (IgG2a). PMN from healthy donors were stimulated in vitro by conditioned medium of 5637, which was shown not to alter Fc receptor expression. The results are expressed as mean of % inhibition in the presence of anti-FCγRI antibodies, with the SEM of n = 5 experiments indicated by the error bars. Statistical analysis (* P < .05; ** P < .01) was performed with data on the absolute amounts of lysis (see Results).

Fig 6. Selective ADCC of PMN via FCγRI. Bispecific antibody 520C9 X 22 [Fab β × Fab β] with specificities against the proto-oncogene product HER-2/neu (MoAb 520C9) and FCγRI (MoAb 22) was tested in an 18-hour ADCC assay against SK-ov3 targets. PMN from healthy individuals and G-CSF-receiving patients were compared using the conditioned medium of 5637 for in vitro stimulation of both effector cell populations. In control experiments with an equimolar mixture of unlinked Fab β fragments of the parental antibodies, only marginal tumor cell lysis (less than 20% of bispecific ADCC) was observed. The results are presented as the mean ± SEM of n = 6 pairs of donors. The differences between control PMN and G-CSF PMN were statistically significant (P < .05).

PMN significantly higher on PMN from G-CSF-treated patients compared with control PMN. 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 precursors 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 FCγRIα, FCγRIβ, and FCγRIε) have been identified that encode four transcripts for the high-affinity receptor for IgG (FCγRI; CD64). RT-PCR analysis was performed using FCγRIα- and FCγRIβ-specific primers, respectively, to see whether also FCγRIβ and FCγRIε transcripts were present in neutrophils during G-CSF therapy. These experiments clearly showed transcription of both the membrane bound isoforms, FCγRIα and FCγRIβ2 (Fig 3). In the murine system, the three-domain FCγRI (corresponding to hFCγRIα) bound IgG with high affinity, but the two-domain receptor form did not.

Therefore, all three domains seem necessary for high-affinity binding. Up to now, the biologic role of the "two-domain" hFCγRIβ2 molecule, as well as of the two soluble forms (hFCγRIβ1 and hFCγRIε), 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 presentation4; 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-FcγR antibody-producing hybridomas as targets in reverse ADCC.3 In these assays, which most selectively engage distinct classes of FcγR, FcγRI, after in vitro induction by IFN-γ, also mediates 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 upon in vivo application of G-CSF. The most abundantly expressed FcγR on PMN (FcγRIIb) is a phosphatidylinositol (PI)-anchored molecule1,2 that lacks a transmembrane portion and, therefore, is supposed to have limited capacity to transmit intracellular signals. Accordingly, FcγRIIb did not mediate cytotoxicity in bispecific or reverse ADCC assays,3 but blocking antibodies to FcγRIII were reported to inhibit lysis of specific tumor targets.42

The biologic role of FcγRI, eg, in antigen presentation,41 and in the pathogenesis or management of infections42,44 has been addressed in recent studies. In addition, FcγRI is an interesting molecule for the therapeutic approach of targeted cytotoxicity.14 As shown here, G-CSF-activated neutrophils can kill a variety of solid tumor cell lines, using clinically interesting MoAbs to the proto-oncogene products erbB2/HER-2 or the EGF receptor. In addition, lysis of freshly obtained tumor cells by PMN was also shown by Cemelcil et al.15 The findings reported here further support the view that a combination of G-CSF and MoAbs to tumor-related epitopes may be effective in cancer therapy.

ACKNOWLEDGMENT
We are indebted to Drs. M.W. Fanger and P.M. Guyre (both Dartmouth Medical School, Lebanon, NH) for generously providing Fc receptor and bispecific antibodies, and for stimulating discussions and critically reviewing the manuscript. We thank Dr J.E. Landegent (University of Leiden, The Netherlands) for expert help with the c-fms PCR experiments. Dr Kwan-Sa You (Medarex, Lebanon, NH) for expert preparation of bispecific antibodies. Dr G.A. Luckenbach (Merck, Darmstadt, Germany) for donating cell lines and antibodies, and T. Ernst, B. Bock, and Ch. Borsum for excellent technical support.

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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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