Recombinant Granulocyte Colony-Stimulating Factor Administration to Healthy Volunteers: Induction of Immunophenotypically and Functionally Altered Neutrophils Via an Effect on Myeloid Progenitor Cells

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We performed a detailed kinetic study on the in vivo effect of a single subcutaneous dose of granulocyte colony-stimulating factor (G-CSF; 300 μg) in four healthy individuals on the expression and function of neutrophil Fcγ receptors (FcγR). G-CSF did not induce FcγRI (CD64) on circulating neutrophils. However, neutrophils newly formed in response to G-CSF were FcγRI positive and were able to perform antibody-dependent cellular cytotoxicity in an FcγRI-dependent way. FcγRII (CD32) expression was not changed significantly. FcγRIII (CD16, phosphatidylinositol-linked) expression, slightly increased immediately (30 minutes) postinjection, was found to be strongly decreased on the newly formed population. For comparison, we studied the expression of the PI-linked proteins leukocyte alkaline phosphatase (LAP) and CD14. Intracellular levels of LAP mirrored the biphasic expression pattern as membrane-bound FcγRIII. In contrast, CD14 expression on neutrophils was initially constant, followed by high levels on the newly formed neutrophils. Soluble CD14 levels were found to be elevated transiently, whereas peak levels of soluble FcγIII were observed as late as 6 days postinjection. In conclusion, we have shown that G-CSF results in an immunophenotypically and functionally altered neutrophil population for an important part as a result of its effect on myeloid precursor cells.

GRANULOCYTE colony-stimulating factor (G-CSF), first described and cloned in 1986,[1,2] is a key hormone for the proliferation and differentiation of committed myeloid progenitor cells of the neutrophil granulocytic lineage. G-CSF also enhances mature neutrophil effector functions.[3,4] In clinical studies, G-CSF has been used for the improvement of circulating neutrophil counts and the reduction of infection.[5] Neutrophils from patients treated with G-CSF have been described to be phenotypically and functionally normal.[4] However, shortly after in vivo G-CSF administration, peripheral blood neutrophils were found to exhibit an increased O_2^- radical production[6-8] as well as increased membrane expression of C3bi receptor (CD11b).[7,9] In addition, during G-CSF therapy the high-affinity receptor for IgG (hFcγRI, CD64 antigen) was detected on circulating neutrophils.[10] This finding was surprising because G-CSF does not induce FcγRI on mature neutrophils in vitro.[11,12] In a previous in vitro study we found that under the influence of G-CSF, committed myeloid progenitor give rise to FcγRI-positive neutrophils.[13] The expression of the phosphatidyl inositol (PI)-linked FcγRIII (CD16) was found to be strongly decreased on these cells. In the present study, we examined in detail the kinetics of Fcγ-receptor expression and function in response to a single dose of G-CSF (300 μg, subcutaneously [sc]) in four healthy volunteers. The results were correlated with the simultaneously measured expression of two other PI-linked proteins, CD14 and LAP, and plasma levels of soluble CD14 and CD16. Our data show that G-CSF results in a profound and long lasting change in phenotype and function of circulating neutrophils and that this is mainly the result of its effect on myeloid progenitor cells.

MATERIALS AND METHODS

Administration of G-CSF to healthy volunteers: Study design. A single dose of G-CSF (Neupogen; Amgen, Thousand Oaks, CA, 300 μg sc) was administered to healthy volunteers (informed consent, according to the rules of our hospital). One woman and 3 men, age 26 to 41 years, participated in the study. Medical history, physical examination, and routine laboratory investigation were completely normal in all subjects. They did not use medication, were nonsmokers, and had no febrile disease in the month before the study. No clinical side effects were observed, neither immediately nor postinjection.

Venous blood samples (EDTA anticoagulated) were obtained immediately before the administration of G-CSF, 5, 15, and 30 minutes and 1, 2, 4, 8, 12, 24, 48, 72, 96, and 144 hours thereafter. Additional samples were obtained 8, 10, 12, and 20 days postinjection. Total leukocyte counts and differential counts were determined with H1 system (Technicon Instruments, Tarrytown, NY). Leukocyte differential counts (morphologic characterization of Jenner-Giemsa stained slides) and deter nination of leukocyte alkaline phosphatase (LAP) score were performed manually on smears of nonanticoagulated blood. For soluble antigens, EDTA plasma was immediately stored at −20°C for subsequent determination.

Immunophenotypical analysis. One peripheral EDTA blood sample was used immediately for flowcytometric analysis: Erythrocytes were lysed by diluting (1:5, vol/vol) peripheral blood with ice-cold isotonic NH_4Cl solution (155 mmol/L NH_4Cl, 10 mmol/L $KHCO_3$, 0.1 mmol/L EDTA, pH 7.4) for 10 minutes. The cells were centrifuged in the cold and the residual erythrocytes were lysed during 3 more minutes. The remaining white blood cells were washed twice in cold PBS/BSA 0,2% (wt/vol) and analyzed by flowcytometry (FACScan, Becton Dickinson, San Jose, CA). Indirect immunofluorescence was performed according to standard techniques, using a panel of murine monoclonal antibodies (MoAbs) against monomyeloid antigens. After preincubation on ice with normal human IgG (Central Laboratory of the Red Cross Blood

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Transfusion Service, Amsterdam, The Netherlands; 1 mg/mL, 10 minutes), the cells were incubated with appropriate dilutions of MoAbs in PBS supplemented with 0.2% BSA (wt/vol) and 0.1% (wt/vol) NaN_3. Binding of the MoAbs was visualized with fluorescein isothiocyanate (FITC)-labeled polyclonal goat-antimouse Ig [F(ab')2]. In the blood samples, neutrophils were easily discriminated from lymphocytes and monocytes by their characteristic forward/sideward scatter. Measurement was made after live gating on neutrophils. In this way, phenotypical changes caused by isolation procedures could be circumvented.[14] The following antibodies were used: CLB irrelevant murine control MoAbs of the IgG1 and IgG2a subclass, CLB HLA class-II (HLA-DR), W6/32 (HLA Class-I), CLB-B2.12 (CD11b), CLB mon-gran/2 (CD13), CLB mon/1 (CD14), CLB FcR-gran/1,[15] CLB-gran/11, 1D3, GRM1 and 3G8[16] (CD16), IV.3[17] and AT10[18] (CD32), HPCA-1 (CD34), CLB-T200/1 (CD45), 197,[19] 22 and 32[20] (CD64), B 13.9 (CD67), and FITC-conjugated goat-antimouse Ig [CLB G26M17F; F(ab')2 FITC]. The 32, IV.3 and 197 MoAbs were from Medarex (West Lebanon, NH). 1D3 was obtained from Dr J.D. Griffin (Dana-Farber Cancer Institute, Boston, MA) and GRM1 from Dr F. Garrido (Hospital Virgen de las Nieves, Granada, Spain). HPCA-1 was obtained from Becton Dickinson. All other MoAbs were produced in our own laboratory and were clustered during the international workshops on leukocyte differentiation antigens.

Antibody-dependent cellular cytotoxicity. From peripheral blood samples (EDTA), neutrophils were carefully purified as described[21] for functional analysis. Antibody-dependent cellular cytotoxicity was determined as described.[22] Briefly, Rh positive erythrocytes were labeled with ^{51}Cr (744 μCi/10^9 cells; Amersham Intl, Amersham, UK) for 60 minutes at 37°C. ^{51}Cr-labeled Rh positive erythrocytes (0.01 mL; 1.0×10^9/mL in PBS) was incubated with 0.01 mL of medium, optimal concentration of standard human anti-D serum or AB serum (CLB) for 60 minutes at 37°C under continuous shaking. After the incubation, the cells were washed three times and resuspended in RPMI 1640 10% (vol/vol) fetal bovine serum at a concentration of 3×10^6 cells/mL. Equal volumes (0.05 mL) of a suspension of labeled and sensitized erythrocytes (3×10^6 cells/mL) and of a suspension of neutrophils (12×10^6 cells/mL) were added to round bottom microtiter plates (Costar, Cambridge, MA). The plates were centrifuged at 500*g* for 5 minutes at room temperature and then incubated for 90 minutes at 37°C and 5% CO_2. The osmolarity of the medium was lowered to that of 0.5% NaCl by addition of 0.1 mL of a hypotonic salt solution (0.1% NaCl) to stimulate ^{51}Cr release from the damaged erythrocytes. Immediately afterwards, the plates were centrifuged for 5 minutes at 500*g* and 0.2 mL of the supernatants was harvested with a supernate-harvesting system (Flow Laboratories, Irvine, CA) and counted for ^{51}Cr activity. The total amount of radioactivity in the red blood cells was measured after lysis with 0.15 mL of a 5% (wt/vol) solution of saponin in distilled water. Sensitized erythrocytes incubated with medium alone, unsensitized erythrocytes with effector cells, and AB-serum–sensitized erythrocytes with effector cells were used as a control for the spontaneous release of radioactivity. The spontaneous release under these conditions never exceeded 4% of the maximal release. The percentage of lysed cells was calculated as: 100(E − S)/TC, in which E is the experimental release, S the spontaneous release, and TC the saponin release. All tests were performed in triplicate.

Determination of LAP activity in neutrophils. Blood smears were dried in air, fixed with ice-cold 10% formaline methanol (vol/vol) for 1 minute, and then incubated with a solution containing Fast-Blue (Pharbill, Rotterdam, The Netherlands) for 15 minutes. After incubation, blood smears were stained (Jenner-Giemsa). Neutrophils showed alkaline phosphatase activity as blue deposits. The degree of activity in each cell was rated according to the number of precipitated blue granules in the plasma. The normal range of score in our laboratory was 0.2 to 0.5.

sCD14 immunoassay. sCD14 levels were determined by enzyme-linked immunosorbent assay (sandwich ELISA) using specific MoAbs directed against nonoverlapping epitopes on CD14. Both CD14 MoAbs were produced in our own laboratory. The purified MoAb CLB mon/1 (CD14) was coated at 4°C (5 μg/mL in phosphate-buffered saline [PBS] 100 μL/well) on flat-bottom microtiter plates (Costar Europe, Badhoevedorp, The Netherlands). All subsequent incubations were in 100-μL vol at 4°C. The plates were washed with PBS, 0.05% (vol/vol) Tween 20 (PT; Baker Chemical Co, Philipsburg, NJ) and nonspecific binding was blocked with PBS 0.2% Tween 20 (vol/vol) and 0.4% gelatine (wt/vol). Serial dilutions of EDTA plasma (diluted in Tris-buffer pH 8.0) were added to the plates for 1 hour. The plates were washed three times in PT and incubated for 1 hour with biotinylated purified MoAb 77 (CD14) (5 μg/mL). Plates were washed and incubated with streptavidin-peroxidase (100 ng/mL; Sigma, St Louis, MO) for 60 minutes. After the final washing step, 100 μL of substrate containing 0.4 mg/mL o-phenylenediamine dihydrochloride (Sigma) was added in 0.05 mol/L phosphate-citrate buffer pH 5.0 containing 0.003% H_2O_2. This reaction was stopped by the addition of equal volumes of 4 mol/L H_2SO_4 to the wells. The absorbance at 450 nm was measured in a Titertek Multiscan Elisa reader (Flow Laboratories, Zwanenburg, the Netherlands). The reactivity of the sample was compared to serial dilutions of pooled plasma from normal donors. The value of the sCD14 was expressed as percentage of the amount of sCD14 in pooled plasma from 52 healthy donors, arbitrarily set at 100 U (mean sCD14 concentration in 52 healthy donors is 99 ± 27 U, mean ± SD).

sCD16 radioimmunoassay. Soluble FcγRIII was determined by a radioimmunoassay essentially as described before.[23] In short, CLB FcR gran/1 was coupled to CNBr-activated Sepharose 4B (10 mg of protein to 1 g of Sepharose). The Sepharose beads were diluted to 0.1% (wt/vol) in PBS containing EDTA (10 mmol/L) and Tween 20 (0.1%, vol/vol). Five hundred microliters of this Sepharose suspension were incubated with 40 μL, 20 μL, or 10 μL of plasma, or with serial dilutions of the pooled plasma from 80 donors (160, 80, 40, 20, 10, 5, 2.5, and 1.25 μL), for 16 hours at room temperature by head-over-head rotation in 2-mL polystyrene tubes. Each test was performed in triplicate. The Sepharose beads were then washed five times with 1.5 mL of saline and incubated for 5 hours at room temperature with ^{125}I-labeled FcγRIII MoAb BW209/2 (Dr R. Kurrle, Behringwerke AG, Marburg, Germany), dissolved in PBS containing EDTA (10 mmol/L), Tween 20 (0.1%), and 5% normal goat serum. Subsequently, the beads were washed five times with saline, and the bound radioactivity was counted in a gamma counter. A calibration curve was made from serial dilutions of pooled plasma, and the value of the sFcγRIII in a volunteers plasma was expressed as percentage of the amount of sFcγRIII in the normal plasma pool. The last amount was arbitrarily set at 100 U (mean sFcγRIII concentration in 175 healthy donors is 97 ± 35 U, mean ± SD).

Northern blot analysis. Northern blot analysis was performed as described.[24] Briefly, RNA was isolated by dissolving the isolated neutrophils in 4 mol/L guanidine thiocyanate and centrifuging through 5.7 mol/L cesium chloride. RNA was blotted onto Gene Screen Plus membrane filters (NEN Research Products, Boston, MA). The blots were hybridized with a *Hin*dIII/*Eco*RI fragment of cDNA FcγRIII.[25] Probes were labeled by random priming. Quantification of FcγRIII mRNA was performed on a slot-blot hybridization assay in six twofold dilutions. Hybridization with a probe for α-actin was used as an internal standard. The spots were analyzed by densitometry.

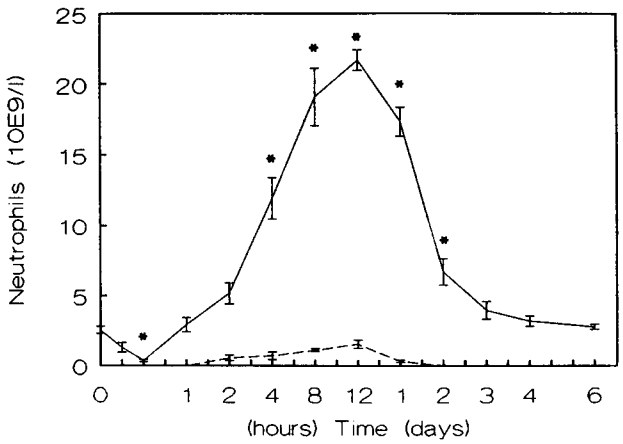

Fig 1. Mean (± SEM) neutrophil counts and numbers of band forms (– – – – –) after a single dose G-CSF (300 μg, sc) to four healthy individuals. Asterisks indicate statistical significance ($P < .05$)

Statistical analysis. Ninety-five percent confidence intervals (CIs) are indicated as: 95% CI. Student's *t*-tests were performed for paired differences.

RESULTS

Peripheral blood leukocyte counts. Baseline leukocyte counts of all four individuals were within normal limits (Fig 1). G-CSF administration was associated with a biphasic change in total leukocyte counts, mainly because of a change in the number of circulating neutrophils. Neutrophils decreased from $2.37 \pm 0.26 \times 10^9$/L at baseline to $0.38 \pm 0.09 \times 10^9$/L (95% CI: 0.1 to 0.66×10^9/L) at 30 minutes postinjection. The decrease in neutrophil count was already apparent after 15 minutes. The initial neutropenia was followed by a sharp increase in neutrophil counts, with a peak level at 12 hours (95% CI 19.3 to 24.1×10^9/L), returning to normal within 96 hours. Differential counts showed a moderate left shift at 4 to 12 hours postinjection (Fig 1; neutrophilic band levels at 12 hours: 95% CI 1.31 to 1.85×10^9/L). More immature myeloid cells (as determined by morphologic analysis) and progenitor release (measured immunophenotypically with MoAb HPCA-1, CD34) could not be detected (data not shown). No toxic neutrophil granulation was observed by morphologic examination. Monocyte counts (at the onset $0.33 \pm 0.03 \times 10^9$/L) were significantly elevated at 12 and 24 hours postinjection (95% CI 0.55 to 1.47 and 0.56 to 0.98×10^9/L, respectively), whereas lymphocyte, eosinophil, basophil, and thrombocyte counts did not change significantly during the observation period (data not shown).

Expression of activation markers on neutrophils shortly after in vivo administration of G-CSF. Because G-CSF administration is associated with a rapid decline in neutrophil counts, we analyzed neutrophils with respect to the activation markers CD13, CD45, CD11b, and CD67. The decrease in neutrophil count (30 minutes postinjection) coincided with upregulated levels of these antigens (95% CI CD13: 101% to 177%; CD45: 113% to 173%; CD11b: 110% to 146%; CD67: 118% to 189%; range-uncorrected mean fluorescence intensities preinjection for four individuals: CD13: 67 ± 4; CD45: 30 ± 3; CD11b: 195 ± 20; CD67: 41 ± 4 arbitrary units), returning to baseline levels after 4 hours postinjection. Thereafter, no significant changes in expression of these antigens were observed. As a control, HLA Class-I expression on neutrophils was found to remain unchanged (data not shown).

Fcγ receptor expression on circulating neutrophils after in

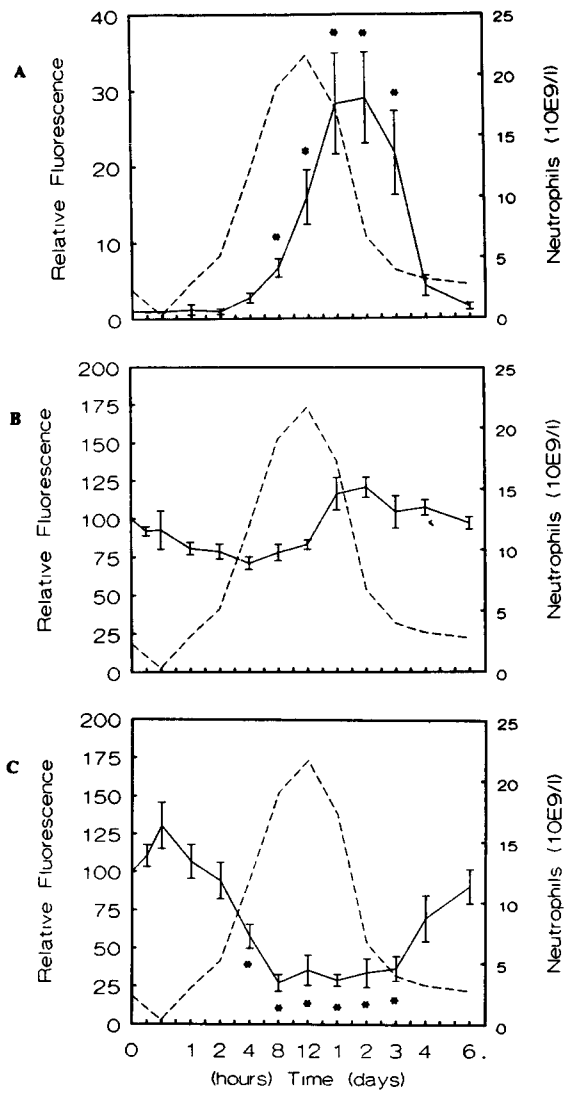

Fig 2. Effect of G-CSF (300 μg sc) on (A) FcγRI (CD64, MoAb 32), (B) FcγRII (CD32, MoAb IV.3), and (C) FcγRIII (CD16, 3G8) expression on circulating neutrophils measured by flow cytometry. Expression as relative fluorescence intensity (preinjection expression intensities of FcγRII and FcγRIII were set at 100%). The mean ± SEM for four individuals is shown. Range-uncorrected mean-fluorescence intensities preinjection for four individuals: MoAb 32: 9 ± 0.5 arbitrary units; MoAb IV.3: 175 ± 19; MoAb 3G8: 843 ± 141. Asterisks indicate statistical significance ($P < .05$). The dashed line represents the mean number of circulating neutrophils in response to G-CSF in four individuals.

vivo application of a single dose G-CSF. Upon administration of G-CSF the circulating neutrophils were found to express FcγRI after an 8- to 12-hour time lag. This receptor, normally absent on neutrophils, remained easily detectable up to 72 hours postinjection (Fig 2A). A similar expression pattern was observed using MoAbs 197 and 22 both binding to different epitopes on FcγRI[19] (data not shown). Using different CD32 MoAbs (IV.3 and AT10) slightly altered FcγRII expression was found, although at no point statistically significant (Fig 2B: MoAb IV.3). In contrast, FcγRIII was mildly upregulated at 30 minutes (95% CI 85% to 179% compared with baseline levels) concomitant with the initial drop in neutrophil count. This initial upregulation was followed by a strongly reduced expression (Fig 2C, 95% CI 24 hours: 17% to 41%; 48 hours: 4% to 63%; 72 hours: 10% to 61%). This biphasic expression pattern was observed with all CD16 MoAbs (CLB FcR gran/1, CLB gran/11, GRM1 and 1D3, data not shown).

After in vivo application of G-CSF newly generated neutrophils perform antibody-dependent cellular cytotoxicity. Because neutrophils generated upon in vivo application of G-CSF were characterized by an altered Fcγ Receptor profile, we analyzed these cells for their capacity to perform antibody-dependent cellular cytotoxicity. As shown in Fig 3A, before G-CSF application, peripheral blood neutrophils lack the capacity to kill RhD sensitized erythrocytes. However, upon administration of G-CSF, neutrophils efficiently kill the target cells. This antibody-dependent cellular cytotoxicity (ADCC) parallels with FcγRI expression and was blocked by MoAb 197 (CD64), whereas MoAb IV.3 (CD32 $F(ab')_2$, CD32) and CLB Fcgran/1 [CD16 $F(ab')_2$, CD16] had no effect (Fig 3B).

Newly formed neutrophils strongly express CD14 and LAP. With respect to LAP content (Fig 4A), a marked decrease was observed within 30 minutes (95% CI: 0.01 to 0.24). After 4 hours, the initial decrease in LAP content was followed by a strong increase reaching a peak level at 48 hours (95% CI: 1.68 to 2.02) step-by-step returning to baseline levels (0.31 ± 0.04). As shown in Fig 4B, circulating neutrophils exhibit a twofold increase in CD14 expression from 8 to 48 hours postinjection (95% CI 12 hours: 146% to 247%; 24 hours: 147% to 254%; 48 hours: 132% to 203%), gradually returning to baseline levels.

sCD14 and sCD16 levels upon in vivo application of a single-dose G-CSF. Both CD14[26] and CD16[23] can be detected in soluble form in plasma. In addition to measurement of neutrophil membrane-bound CD14 and CD16, we determined plasma levels of these antigens using immunoassays. As shown in Fig 5A, sCD14 levels initially remained relatively constant. Significant elevated levels were measured between 8 and 24 hours after G-CSF application (95% CI 8 hours: 117% to 161%; 12 hours: 122% to 162%; 24 hours: 132% to 152%). In contrast to neutrophil membrane-bound CD16, sCD16 levels were significantly elevated 4 hours after G-CSF. Thereafter, sCD16 levels remained relatively constant, but peak levels were measured around day 6 (95% CI: 129% to 182%, see Fig 5B).

Northern blot analysis. To analyze the effect of G-CSF on mRNA for FcγRIII in newly formed neutrophils, Northern blot analysis was performed on mRNA obtained from neutrophils before G-CSF application and 24 and 48 hours postinjection. As shown in Table 1, no differences in FcγRIII-mRNA levels in the respective neutrophil populations were observed.

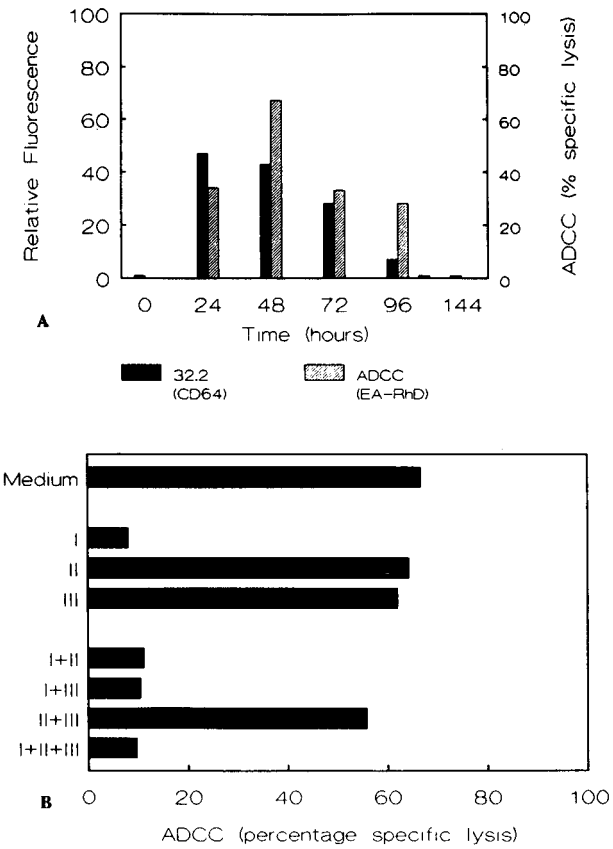

Fig 3. Cellular cytotoxicity of polymorphonuclear cells upon G-CSF administration to healthy volunteers. ADCC of neutrophils preinjection and after administration of G-CSF (300 μg, sc). The results of one representative volunteer of four are shown in relation to FcγRI expression on neutrophils (MoAb 32, relative fluorescence). ADCC is expressed as percentage of specific lysis (see Materials and Methods). (B) Role of FcγRI, FcγRII, and FcγRIII in cellular cytotoxicity of neutrophils. Saturating amounts of blocking antibodies to FcγRI (MoAb 197; whole antibody), FcγRII [MoAb IV.3, $F(ab')_2$] of FcγRIII [MoAb CLB RcR-gran 1, $F(ab')_2$] alone or in combination. Blocking of maximal ADCC at 48 hours postinjection of the representative volunteer is shown and expressed as percentage specific lysis.

DISCUSSION

In the present study we administered a single dose of G-CSF (300 μg sc) to healthy volunteers. As reported by Layton et al,[27] this results in peak levels of G-CSF after about 4 hours. At 24 hours, serum levels are less than 10% of the peak level. As summarized in Table 2, the G-CSF effects can be divided into four different phases. First, the immediate effect (peaking at 30 minutes) reflects G-CSF effects on circulating neutrophils. The observed decrease in neutrophil counts, which has been reported previously,[6,28] is most

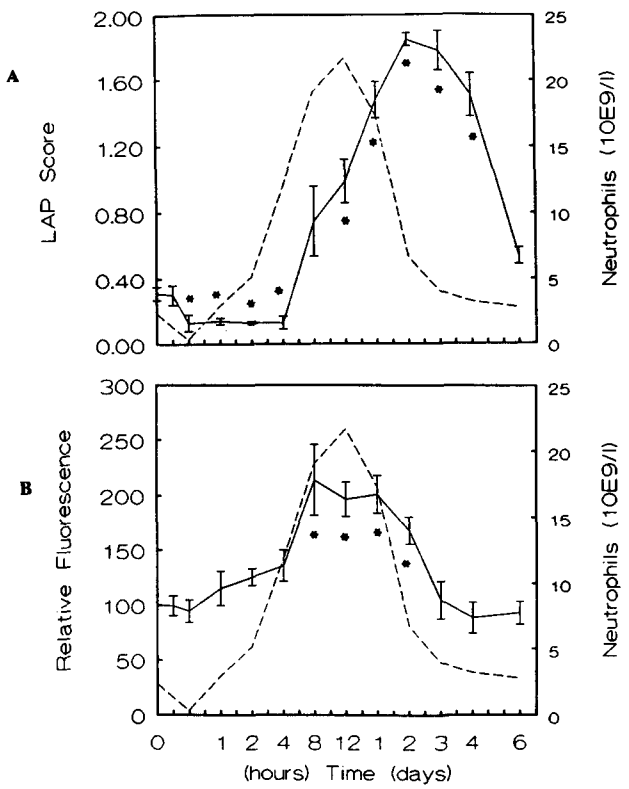

Fig 4. Mean LAP score (A) and CD14 (B) expression of circulating neutrophils after administration of G-CSF (300 μg, sc) to four healthy individuals. LAP scores were determined as indicated in Materials and Methods (normal range of score in our laboratory was 0.2 to 0.5). CD14 expression on neutrophils was determined by flow cytometry and expressed as relative fluorescence intensity (preinjection, expression intensities were set at 100%). The mean ± SEM for four individuals is shown. Range-uncorrected mean-fluorescence intensities preinjection for four individuals: MoAb 8G3 (CD14): 37 ± 4. The asterisks indicate statistical significance ($P < .05$). The dashed line represents the mean number of circulating neutrophils in response to G-CSF in four individuals.

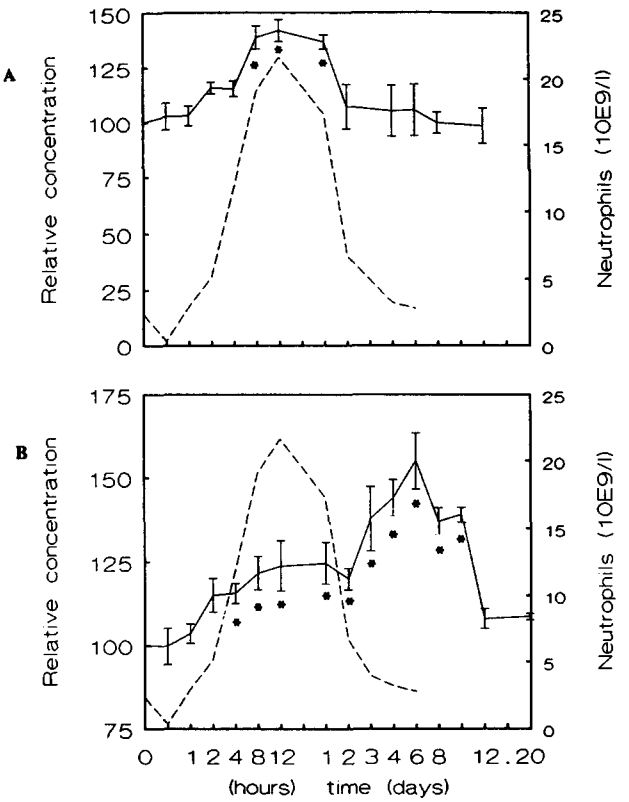

Fig 5. Mean (± SEM) plasma concentration of sCD14 and sCD16 after a single-dose G-CSF (300 μg, sc). Plasma concentrations were measured as indicated in Materials and Methods. Asterisks indicate statistical significance ($P < .05$). The dashed line represents the mean number of circulating neutrophils in response to G-CSF in four individuals.

likely caused by adhesion of activated neutrophils to vascular endothelium. Indeed, 30 minutes postinjection, circulating neutrophils exhibit an increased expression of antigens considered to be associated with neutrophil activation such as CD13 (aminopeptidase N), CD45 (membrane-associated tyrosine phosphatase),[14,29,30] CD11b (C3bi receptor),[7,9,31] and of the PI-linked protein CD67.[32] The level of activation as measured by flowcytometry may very well be underestimated due to margination of the large majority of the neutrophils. The second phase is characterized by a gradual increase in neutrophil numbers reaching peak levels at 12 hours postinjection (early effects). This second phase probably reflects release of storage pool neutrophils. Because G-CSF has been described to reduce the marrow transit time of the maturating myeloid population from 5 days to 1 and to result in 3.2 extra amplification divisions during neutrophil development,[33] the population of neutrophils circulating 24 hours or more after G-CSF application (late effect) consists of newly formed cells, as a result of G-CSF action on myeloid precursor cells. Fourth, effects after a lag time of several (6 or more) days reflect the tissue phase of the neutrophils (very late effects).

We studied the kinetics of the neutrophil Fcγ-receptor profile in response to G-CSF. Upon G-CSF application, we observed FcγRI-positive neutrophils after an 8- to 12-hour lag time. The in vitro ability of G-CSF to induce FcγRI in neutrophils is limited.[13] Therefore, FcγRI-positive neutrophils result from G-CSF action on progenitor cells rather than on mature circulating cells. Previous in vitro studies have shown that G-CSF, when used alone, is able to generate FcγRI-positive neutrophils from highly purified $CD34^+$ bone marrow cells. This effect could not be blocked by addi-

Table 1. Quantification of FcγRIII mRNA by Slot-blot Analysis

mRNA Derived at	Percent of α-Actin mRNA
0 h	93
24 h	91
48 h	86

Hybridization and quantification were performed as described in the Materials and Methods section. Amounts of FcγRIII are relative to α-actin. Data of one volunteer are shown.

Table 2. Effects of G-CSF In Vivo to Healthy Volunteers

	Immediate	Early	Late	Very Late
Time peak	30 min	8-12 h	24-72 h	6 d
Neutrophil count	↓↓	↑↑↑	↑/N	N
Activation markers				
(CD13, CD45, CD11b, CD67*)	↑	N	N	N
Fcγ receptor profile				
FcγRI	N	N/↑	↑↑↑	N
FcγRII	N	N	N	N
FcγRIII*	↑	↓↓	↓↓↓	N
LAP score*	↓↓↓	↑↑	↑↑↑	N
CD14*	N	↑↑	↑↑	N
Soluble antigens				
sCD16	N	↑	↑	↑↑
sCD14	N	↑↑	↑/N	N

Abbreviation: N, no effect.
* PI-linked antigen.

tion of MoAb against interferon γ.[13] Thus, although not totally excluded, it is not very likely that the in vivo G-CSF effects are mediated by interferon γ. The newly formed neutrophils were shown capable of FcγRI-mediated ADCC. Isaacs et al[34] described high FcγRI-mRNA expression in neutrophils from patients treated with G-CSF. Repp et al[10] reported that FcγRI positive neutrophils from cancer patients treated with G-CSF display cytotoxicity against opsonized Daudi lymphoma cells, although a role of FcγRI was not shown with inhibitory MoAb. FcγRII expression on the neutrophils was not changed significantly in response to G-CSF. In contrast, FcγRIII expression showed remarkable changes. Immediately postinjection, activated neutrophils expressed slightly upregulated levels of FcγRIII, possibly from a stored protein pool. Early and, to a greater extent, late after G-CSF administration, FcγRIII expression on neutrophils was strongly decreased. Several possible explanations can be considered for the decreased FcγRIII expression. (1) Decreased FcγRIII expression might reflect egress of immature myeloid cells, because strongest FcγRIII expression is found on mature segmented cells.[35] However, this is highly unlikely because early postinjection, a mild left shift was observed (Fig 1). (2) Shedding of FcγRIII might contribute to decreased expression on neutrophils. In early postinjection we measured slightly increasing levels of sFcγRIII, probably reflecting release from circulating activated neutrophils. However, during the late phase (24 to 72 hours), newly generated, FcγRIII-low neutrophils do not exhibit an 'activated phenotype' and soluble-FcγRIII levels remain relatively constant. Therefore, we conclude that release does not account for the markedly decreased FcγRIII expression. Interestingly, peak levels of sFcγRIII were measured as late as 6 days postinjection (very late effect of G-CSF). This finding is compatible with our previous observations that the concentration of sFcγRIII is related to the total mass of neutrophils in the body and is not influenced by shifts between storage, marginating or circulating pool.[36] The abundant production of neutrophils is reflected by increased soluble-FcγRIII levels after a lag time of several days caused by migration of neutrophils into tissue, subsequent release of sFcγRIII and finally appearance of the soluble protein in the peripheral blood. (3) Newly formed neutrophils have a different distribution between membrane-bound and intracellular FcγRIII. This possibility is supported by our demonstration that these newly generated neutrophils contain normal levels of mRNA for FcγRIII (Table 1). Moreover, upon in vitro activation (eg, density gradient centrifugation) the membrane-bound FcγRIII expression levels on newly generated neutrophils rapidly reach normal levels, indicating the presence of preformed FcγRIII in the cells. The exact intracellular localization of FcγRIII is not yet known. The presence of intracellular FcγRIII has been reported by Jost et al.[37] Tosi and Zakem[38] detected intracellular storage of FcγRIII predominantly in alkaline phosphatase-containing fraction. Therefore, the so-called secretory vesicles might be the place of intracellular localization.[39]

To investigate whether the decreased membrane-expression of FcγRIII was a general phenomenon for PI-linked proteins, we also studied the expression of two other PI-linked proteins, LAP[40] and CD14.[26] Also for LAP, a biphasic pattern was observed. Immediately after activation, neutrophils show strongly decreased intracellular LAP content (Fig 4A), whereas strongly enhanced intracellular LAP content was detected early and late postinjection. LAP is located intracellulary in highly mobilizable vesicles and is translocated to the plasma membrane after in vitro stimulation.[39] G-CSF has been described to upregulate mRNA levels of LAP in myeloid cells[41,42] and to increase LAP content in neutrophils.[43,44] Because the biphasic expression pattern of intracellular LAP mirrors that of membrane-bound FcγRIII, a shared intracellular localization might be possible. In contrast to FcγRIII and LAP, neutrophil membrane CD14 levels did not change immediately postinjection. A twofold increase in CD14 expression on neutrophils was detected early as well as late postinjection. This is in agreement with a previous study where we showed that neutrophils generated in a G-CSF stimulated in vitro model of myelopoiesis had a surprisingly high expression level of CD14.[45] In addition, sCD14 levels were transiently elevated up to 48 hours postinjection. Whether sCD14 originates from monocytes of neutrophils is not yet clear. CD14 expression on monocytes remained unchanged (data not shown). CD14 has been shown to serve as a receptor for the complex of LPS and LPS-binding protein,[46] whereas sCD14 enables responses to LPS by cells that do not express CD14.[47] We postulate that increased expression of CD14, both neutrophil membrane-bound and soluble, might provide an extra defense mechanism against bacterial products.

In conclusion, we have shown that application of a single dose of G-CSF results in both immunophenotypically and functionally altered neutrophils. These newly formed neutrophils are well equipped against bacterial infections in terms of FcγRI expression, FcγRI-dependent ADCC and strong-surface CD14 expression. The biologic significance of the decreased surface-expression of FcγRIII and the high intracellular LAP content remains to be elucidated. The effect of G-CSF is long lasting and is mainly caused by effect

of G-CSF on myeloid precursor cells. Elevated levels of G-CSF levels have been found under several hematologic conditions and after cytoreductive therapy,[48] during bacterial infections[49] and after bone marrow transplantation.[50] This study emphasizes the pivotal role of G-CSF in regulating the nonspecific defense.

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