DURING RECENT YEARS granulocyte colony-stimulating factor (G-CSF) has been used successfully in clinical studies to accelerate the recovery of neutrophil counts after cytostatic therapy and thus to reduce the occurrence of infections. In addition to its effect on the production of neutrophils, G-CSF also modulates several neutrophil functions. In an initial study in healthy volunteers, we focused on the characteristics of the neutrophils that were newly generated as a result of G-CSF on myeloid precursor cells. We found that G-CSF induces the production of phenotypically and functionally altered neutrophils with respect to Fcy receptor profile and antibody-dependent cellular cytotoxicity, respectively. As G-CSF acts also on mature neutrophils, we analyzed the immediate effects of G-CSF administration on the circulating neutrophils.

It is known that G-CSF primes neutrophils for superoxide production stimulated by FMLP when used in vitro experiments and when applied in vivo. Furthermore, in vitro exposure of neutrophils to G-CSF increases the affinity of the adhesion protein L-selectin for its carbohydrate ligand. This is followed by a decrease in surface expression of L-selectin, due to shedding. Finally, in vitro G-CSF induces an increased expression of the β2-integrin CD11b, which is also found shortly after G-CSF administration in vivo. Apparently, G-CSF induces the mobilization of the intracellular pool of CD11b.

After administration of G-CSF an immediate drop in neutrophil counts has been reported, which is most likely the result of neutrophil activation. In vitro activation of neutrophils is reflected by mobilization of the different intracellular neutrophil compartments. Of these, the secretory vesicles form the fastest mobilizable compartment. The exocytosis of specific granules can be triggered in primed neutrophils by several neutrophil agonists, whereas degranulation of azurophil granules is only achieved in the presence of cytochalasin B, which interferes with the function of the cytoskeleton. There are no reports on degranulation of neutrophils after G-CSF exposure. Therefore, we analyzed in healthy volunteers the degranulation of circulating neutrophils upon in vivo application of G-CSF.

In four healthy volunteers, we analyzed in detail the immediate in vivo effects on circulating neutrophils of subcutaneous administration of 300 μg of granulocyte colony-stimulating factor (G-CSF). Neutrophil activation was assessed by measurement of degranulation. Mobilization of secretory vesicles was shown by a decrease in leukocyte alkaline phosphatase content of the circulating neutrophils. Furthermore, shortly postinjection, FcyRIIia was found to be upregulated from an intracellular pool that we identified by immunoelectron microscopy as secretory vesicles. Intravascular release of specific granules was shown by increased plasma levels of lactoferrin and by upregulation of the expression of CD66b and CD11b on circulating neutrophils. Moreover, measurement of fourfold elevated plasma levels of elastase, bound to its physiologic inhibitor α1-antitrypsin, indicated mobilization of azurophil granules. However, no expression of CD63, a marker of azurophil granules, was observed on circulating neutrophils. G-CSF—induced mobilization of secretory vesicles and specific granules could be mimicked in whole blood cultures in vitro, in contrast to release of azurophil granules. Therefore, we postulate that the most activated neutrophils leave the circulation, as observed shortly postinjection, and undergo subsequent stimulation in the endothelial microenvironment, resulting in mobilization of azurophil granules. Our data demonstrate that G-CSF should be regarded as a potent immediate activator of neutrophils in vivo.

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MATERIALS AND METHODS

Administration of G-CSF to healthy volunteers: Study design. A single dose of G-CSF (Neupogen, 300 μg subcutaneously; Amgen, Thousand Oaks, CA) was given to healthy volunteers (with informed consent, according to the rules of our hospital, Academic Medical Center). Four healthy volunteers (age range, 26 to 41 years; one woman, three men) participated in the study. Medical history, physi-
cal examination, and routine laboratory investigation were normal in all subjects. They did not use any medication, did not smoke, and had no febrile disease in the month before the study. The volunteers were under medical observation during the first day of the trial.

Venous blood samples (EDTA anticoagulated) were obtained immediately before the administration of G-CSF and 5, 15, and 30 minutes and 1, 2, 4, 8, 12, 24, 48, 72, 96, and 144 hours thereafter. Additional plasma samples were obtained 7, 8, 10, 12, and 20 days postinjection. Total leukocyte counts and differential counts were determined with the H1 system (Technicon Instruments, Tarrytown, NY). Leukocyte differential counts (morphologic characterization of Jenner-Giemsa–stained slides) and determination of leukocyte alkaline phosphatase (LAP) scores were performed manually on smears of non-anticoagulated blood. For measurement of released proteins, EDTA plasma was immediately stored at −20°C for subsequent determination.

Measurement of G-CSF plasma levels. G-CSF levels were measured with a commercial enzyme-linked immunosorbent assay (ELISA) test kit (Quantikine Human G-CSF Immunoassay, R&D Systems, Minneapolis, MN), according to the manufacturer’s instructions. Briefly, 100 µL of plasma or dilutions of plasma were incubated for 2 hours in a microtiter plate precoated with an anti-G-CSF monoclonal antibody (MoAb). After washing, bound G-CSF was detected by horseradish peroxidase–labeled polyclonal rabbit anti-G-CSF Ig. The concentration of G-CSF in a plasma sample was determined by comparison with a provided standard of dilutions of recombinant human (rh) G-CSF. The lower detection level of the assay was 10 pg/mL.

Immunophenotypic analysis. Blood samples were used immediately for flow cytometric analysis. Erythrocytes were lysed by diluting (1:5, vol/vol) peripheral blood with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4) for 10 minutes. The cells were centrifuged in the cold, and the residual erythrocytes were lysed for 3 more minutes. The remaining white blood cells were washed twice and resuspended at 15 × 10⁶/ml in cold phosphate-buffered saline (PBS)/bovine serum albumin (BSA), 0.2% wt/vol. Indirect immunofluorescence was performed according to standard techniques, with a panel of murine MoAbs against myeloid antigens. The cells were incubated with appropriate dilutions of MoAb in PBS supplemented with 0.2% BSA (wt/vol) and 0.1% wt/vol Na₂CO₃. Binding of the MoAb was visualized with fluorescein isothiocyanate (FITC)-labeled F(ab')₂ fragments of polyclonal goat IgG against mouse Ig. Flow cytometric analysis was performed with a FACScan (Becton Dickinson, San Jose, CA). 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, phenotypic changes due to isolation procedures were circumvented.¹⁷ The following antibodies were used: CLB irrelevant murine control MoAb of the IgG1 and IgG2a subclass, CLB-B2.12 (CD11b), CLB-FcRgran1²⁰ and 3G8²⁰ (CD16), IV.3 (CD32),²⁰ CLB-435 (CD63),²⁰ B 13.9 (CD66b, formerly named CD67),²⁰ and FITC-conjugated goat-antimouse-Ig [CLB G26M17F; F(ab')₂ FITC]. The 3G8 and IV.3 MoAbs were from Medarex (West Lebanon, NH); all other MoAbs were produced in our own laboratory, and their reactivity was established during the Fourth International Workshop on Human Leukocyte Differentiation Antigens (Vienna, Austria, 1989).

Determination of LAP activity in neutrophils after G-CSF administration in vivo. Non-anticoagulated blood smears were dried in air, fixed with ice-cold 10% formalin methanol (vol/vol) for 1 minute, and then incubated with a solution containing Fast-Blue for 15 minutes. After this incubation, the blood smears were stained according to Jenner-Giemsa. Neutrophils showed alkaline phosphatase as blue deposit. The degree of activity in each cell was rated according to the number of precipitated blue granules in the cytoplasm. The normal range of score in our laboratory is 0.2 to 0.5 as determined in 100 neutrophils.

Whole blood incubations. Freshly drawn sodium-heparin anticoagulated blood was incubated with either titrated concentrations of G-CSF, FMLP (1 µM/L), or PBS in 24-well plates at 37°C. The incubation was ended by dilution of the blood with ice-cold isotonic NH₄Cl solution. Subsequently, the leukocytes were isolated and stained as described in “Immunophenotypic Analysis” and studied by flow cytometry. Plasma was isolated from simultaneously activated whole blood samples. Whole blood incubations with FMLP (1 µM/L) and cytochalasin B (5 µg/mL) for 30 minutes at 37°C were performed to induce maximal release of lactoferrin and elastase.

Activation of neutrophils. The purification and subsequent activation of neutrophils with FMLP (1 µM/L) or titrated G-CSF concentrations was performed as described previously.²⁷ After activation of the cells, the supernatant was harvested, and released FcyRIII was measured in the sFcyRIII radioimmunoassay (see below). The amount of released FcyRIII was expressed relatively to the total FcyRIII content in a lysate of resting neutrophils.

sFcyRIII radioimmunoassay. Soluble FcyRIII levels were determined by a radioimmunoassay (RIA) essentially as described before.²¹ In short, CLB-FcRgran1 coupled to CNBr-activated Sepharose 4B was incubated with plasma for 16 hours at room temperature by head-over-head rotation in 2-mL polystyrene tubes. Bound sFcyRIII was detected by a subsequent incubation (5 hours at room temperature) with ¹²⁵I-labeled FcyRIII MoAb BW209/2 (Dr R. Kurrle, Behringwerke AG, Marburg, Germany). Samples were tested in triplicate. Results were expressed as percentages of sFcyRIII in a pool of 80 plasma samples from normal donors. Dilutions of this pool were tested in each assay as a standard.

Determination of elastase and lactoferrin release. Elastase–α₁-antitrypsin (elastase–α₁AT) complexes and lactoferrin were measured using a RIA as described previously.²⁸ All polyclonal antisera and MoAbs used in these assays were produced in our own laboratory. Briefly, immunoglobulin enriched fractions of a rabbit antiserum against human-neutrophil elastase or MoAb 13.17 (anti-lactoferrin) were coupled to CNBr-activated Sepharose beads and incubated with plasma samples (4 hours). The beads were washed and incubated (16 hours) with ¹²⁵I-labeled MoAb AT15 directed against α₁AT or polyclonal ¹²⁵I-labeled anti-lactoferrin. Levels of elastase–α₁AT complexes and lactoferrin were expressed as nanograms per milliliter with preformed complexes and lactoferrin as standards, respectively.

Immuno-electron microscopy. Leukocytes were isolated as described under immunophenotypic analysis, fixed for 2 hours at room temperature in a graded (2% to 8%) paraformaldehyde (PFA) series in PBS to preserve the antigenicity of FcyRII.²⁹ and pellets in 10% gelatin. Subsequently, the samples were prepared for cryosectioning and immunolabeling as described previously.¹² Double-labeling experiments were performed with mouse MoAb CLB-FcRgran1 (anti-FcyRIII) followed by incubation with rabbit-antimouse Ig and goat-antirabbit Ig conjugated to 5 nm gold (all anti-rabbit gold conjugates were from Amersham Nederland, ’s-Hertogenbosch, The Netherlands). Albumin was detected by rabbit-antihuman albumin (CLB, Amsterdam, The Netherlands), and lactoferrin was detected with rabbit-antihuman lactoferrin (Cappel Laboratories, Cochranville, PA). Both sera were visualized with goat-antirabbit Ig conjugated to 15 nm gold. For the controls, the primary antibody was replaced by a nonrelevant murine or rabbit Ig. After immunolabeling, the cryosections were embedded in a mixture of methacrylate and uranyl acetate. All sections were examined with a Philips CM10 electron microscope (Eindhoven, The Netherlands).

To study the mobilization of secretory vesicles, whole blood was incubated as described above and after 30 minutes of incubation
cytes were treated with a mixture of 0.5% glutaraldehyde and fixed with NEUTROPHIL DEGRANULATION UPON G-CSF.

Sections were incubated with anti-albumin antiserum and 10 nm gold particles on the cell membrane were scored.

Statistical analysis. Student's t-tests were performed for paired differences. A two-sided P value < .05 was considered to indicate a significant difference.

RESULTS

Peripheral blood G-CSF levels and neutrophil counts. Preinjection endogenous levels of G-CSF measured in plasma of two volunteers were 53 pg/mL and 44 pg/mL (Fig 1), which is within the normal range (< 78 pg/mL, n = 48).

At 5 minutes postinjection, 30-fold elevated levels of G-CSF were measured in the plasma sample. Peak levels of G-CSF were measured at 4 hours postinjection (25,000 pg/mL and 44,000 pg/mL), and at 24 hours, G-CSF levels were approximately 300 pg/mL for both donors. Two days postinjection the G-CSF levels had returned to preinjection values. No clinical side effects were observed in the volunteers, neither immediately nor later postinjection. Fifteen minutes after injection of G-CSF the number of circulating neutrophils decreased, with a nadir of 0.38 ± 0.09 × 10⁹/L at 30 minutes (Fig 1). Subsequently, neutrophil counts increased rapidly and remained elevated until 48 hours postinjection, as discussed previously.¹

Expression of activation markers on neutrophils short after in vivo administration of G-CSF. The nadir in circulating neutrophils at 30 minutes postinjection coincided with a significant upregulation of the expression of CD11b (localized in secretory vesicles and specific granules) and CD66b (marker of specific granules) on the remaining neutrophils in the circulation (Fig 2A and 2B, respectively). At this time, peak levels of CD66b were measured, and the expression of CD66b remained elevated until 4 hours postinjection. CD11b was maximally expressed at 2 hours postinjection and returned to baseline levels at 4 hours postinjection. As shown in Fig 2C, the expression of FcyRIII was increased shortly after G-CSF administration, followed by a strong decrease in expression. However, the response proved to be heterogeneous: a significant elevation of FcyRIII expression was found in two donors, whereas the two other donors showed a constant level of expression of FcyRIII until 1 hour postinjection, followed by a decreased FcyRIII expression.

Because activation of neutrophils results in shedding of FcyRIII,²⁷ we determined whether FcyRIII was released in response to G-CSF by measuring the plasma levels of sFcyRIII. Indeed, sFcyRIII levels were increasing during the first hours postinjection in all donors (Fig 2C). Finally, no significant change in expression of CD63 (marker of azurophil granules), FcyRII, or L-selectin was found (data not shown).

Degranulation of neutrophils after G-CSF administration in vivo. The intracellular LAP content (marker of secretory vesicles) of the neutrophils circulating during the neutrophilic phase was found to be significantly decreased. Moreover, during the first 4 hours postinjection LAP values remained at this low level (Fig 2D). Thereafter, a sharp increase in LAP content was observed, probably as a result of increased LAP production, as was discussed in our previous report.³ With respect to degranulation of the two other intracellular neutrophil compartments, G-CSF administration induced within 1 hour a significant rise in plasma levels of both lactoferrin (specific granules) and elastase-α1AT complexes (azurophil granules; Fig 3A and 3B, respectively). The level of lactoferrin (baseline level, 93 ± 11 ng/mL) exhibited a sharp increase and peaked at 4 hours postinjection, showing a 4.2 ± 0.6-fold increase. Plasma levels of elastase-α1AT (33 ± 5 ng/mL before G-CSF administration) peaked at 8 hours postinjection, showing a 4.1 ± 0.3-fold increase. Compared with lactoferrin levels, which had returned to preinjection values at 24 to 48 hours postinjection, the levels of elastase-α1AT returned more gradually to baseline levels. Furthermore, a second transient elevation in the plasma levels of lactoferrin, peaking at 7 days postinjection, was found.

Expression of activation markers on neutrophils after in vitro exposure to G-CSF. The stimulatory effect of G-CSF was studied in vitro by incubation of freshly drawn whole blood with various concentrations of G-CSF (1 to 100 ng/mL). Concomitantly, samples were also incubated with 1 μM FMLP, a concentration known to induce fusion of both secretory vesicles and specific granules with the cell membrane.¹⁶,¹⁷ A time- and G-CSF concentration-dependent upregulation of the expression of CD11b and CD66b was observed (Fig 4A and 4B). Based on these results and on G-CSF plasma levels measured shortly postinjection in vivo (Fig 1), we performed additional whole blood incubations using a G-CSF concentration of 10 ng/mL.

As shown in Fig 4C, G-CSF (10 ng/mL) induced a significant upregulation of the expression of FcyRIII. In con-
Fig 2. Effect of G-CSF administration to four healthy individuals (300 µg subcutaneously) on CD11b (A), CD66b (B), and FcyRlll (CD16; C) expression on circulating neutrophils measured by flow cytometry (——). Expression is depicted as relative fluorescence intensities (preinjection levels set at 100%). The mean ± SEM for four healthy volunteers is shown. Range for uncorrected mean fluorescence intensities preinjection: CD11b, 195 ± 20; CD66b, 41 ± 4; FcyRlll, 843 ± 141 arbitrary units. (C) Levels of sFcyRII1 (mean ± SEM in arbitrary units, ———). (D) LAP content (——). Preinjection LAP scores (0.31 ± 0.03), determined as described in Materials and Methods, were set at 100%. Mean neutrophil counts are depicted as a dashed line (A, B, and D). Asterisks indicate statistical significance (P < .05).

In contrast to the expression of all other antigens studied, nonstimulated neutrophils exhibited a temporarily decreased expression of FcyRlll during the incubation period. The decrease in expression was not reflected by an increase in the level of sFcyRII1 in the plasma that was harvested at the end of the incubation, which argues against shedding of FcyRlll. G-CSF also did not induce shedding of FcyRlll, neither in whole blood cultures nor by incubation of isolated neutrophils.

Fig 3. Mean ± SEM lactoferrin (A) and elastase-α1AT (B) plasma levels in response to G-CSF administration (300 µg subcutaneously) to four healthy volunteers, expressed as relative concentration (——). Mean neutrophil counts are depicted as a dashed line. Asterisks indicate statistical significance (P < .05).
Neutrophils (13% ± 2% of total neutrophil FcyRIII was released without stimulus, 10% ± 3% by G-CSF (10 ng/mL), and 40% ± 4% by FMLP; n = 4).

Expression of CD63 and FcyRIII were not changed significantly by G-CSF (10 ng/mL), whereas the expression of L-selectin decreased significantly by G-CSF to 79% ± 2% of the baseline level (n = 3, data not shown).

Degranulation after in vitro activation of neutrophils by G-CSF. In vitro incubation of whole blood with G-CSF (10 ng/mL) resulted in the release of lactoferrin but not in the release of elastase. After G-CSF activation, lactoferrin levels increased from 83 ± 28 ng/mL to 191 ± 2 ng/mL (1.9 ± 0.01-fold increase, n = 3; Fig 5). FMLP stimulation induced a 14.7 ± 2-fold increase of lactoferrin levels (Fig 5, n = 3). G-CSF did not induce release of elastase, not even when the incubation period was extended to 4 hours and G-CSF concentrations of 100 ng/mL were tested (n = 3, data not shown). In contrast, elastase release was observed in blood samples incubated with either 1 μmol/L FMLP alone or with 1 μmol/L FMLP in combination with 5 μg/mL cytochalasin B (465 ± 42 ng/mL elastase-α1AT per milliliter and 1,340 ± 235 ng/mL, respectively).

Immuno EM studies on the intracellular localization of FcyRIII and the activation of neutrophils upon addition of G-CSF. Albumin is a marker of secretory vesicles detectable by immuno EM23,10 (Fig 6a). We found co-localization of FcyRIII and albumin (Fig 6b) but not of FcyRIII and lactoferrin, a marker of specific granules (Fig 6c). Thus, the intracellular pool of FcyRIII is formed by secretory vesicles.

Fig 4. Neutrophil activation in response to titrated G-CSF concentrations as assayed in whole blood cultures in comparison with FMLP (10^-6 mol/L) and measured by flow cytometry (n = 4). (A) CD11b expression. (B) CD66b expression. (C) FcyRIII (CD16) expression.

Fig 5. Lactoferrin release after activation of neutrophils in in vitro whole blood incubations (n = 3). The change in relative lactoferrin levels in the harvested plasma is depicted (mean ± SEM). Preincubation levels of lactoferrin (83 ± 28 ng/mL) were set at 100%. Medium, G-CSF (---), 10 ng/mL; FMLP (--.--), 1 μmol/L.
Fig 6. Immuno EM of ultrathin cryosections of neutrophils. (a) Localization of albumin after incubation with rabbit-antihuman albumin and goat-antirabbit Ig conjugated to 10 nm gold. Two areas of the same section showing vesicles containing albumin (arrows). (b) Localization of FcγRIII and albumin. FcγRIII was labeled first with MoAb CLBFcRgran1, rabbit-antimouse Ig and goat-antirabbit Ig conjugated to 5 nm colloidal gold, whereas albumin was directly detected with rabbit-antihuman albumin and goat-antirabbit Ig conjugated to 15 nm gold. FcγRIII (5 nm gold) is shown in vesicles (small arrows) in association with albumin (large arrows, 15 nm gold). No labeling was seen on granules or nucleus (n). (c) Localization of FcγRIII and lactoferrin. FcγRIII was labeled as described in (b), and lactoferrin was detected with rabbit-antihuman lactoferrin and goat-antirabbit Ig conjugated to 15 nm gold. Lactoferrin (15 nm gold) was localized in the specific granules (large arrows), whereas FcγRIII labeled with 5 nm gold (small arrows) was localized in vesicles. Nucleus (n). Bars indicate 200 nm. In (a), the morphology of the vesicles is optimal because of the fixation with glutaraldehyde, whereas in (b) and (c) the membrane of the vesicles is hardly visible, because the cells were fixed only with formaldehyde to optimize the staining of FcγRIII.
Table 1. Neutrophil Activation in Response to G-CSF or FMLP: Localization of Albumin

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<th>Medium $^t$ (10 ng/mL)</th>
<th>FMLP $^t$ (1 μM/L)</th>
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<tr>
<td>Secretory vesicles</td>
<td>433</td>
<td>376</td>
<td>211</td>
</tr>
<tr>
<td>Surface membrane</td>
<td>190</td>
<td>675</td>
<td>920</td>
</tr>
<tr>
<td>Large vesicles</td>
<td>7</td>
<td>16</td>
<td>55</td>
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Leukocytes that were isolated after whole blood incubations (30 minutes at 37°C) were fixed and measured by immunogold staining for albumin in frozen thin sections. Micrographs of 20 cell profiles were examined for each incubation.

* Whole blood was fixed immediately after venapunction.
† PBS was added instead of an activator.
‡ Number of positive vesicles.
§ Gold particles on the cell surface membrane.

Furthermore, by semiquantitative EM we analyzed the effect of G-CSF on the mobilization of secretory vesicles. Table 1 shows that after G-CSF incubation a decrease in the number of secretory vesicles was found. The effect of FMLP on secretory vesicle mobilization was more pronounced (Table 1), similar to the results obtained in the experiments on G-CSF— and FMLP—induced upregulation of CD11b and FcγRIII (Fig 4A and 4C). Like FMLP, G-CSF induced the formation of large vesicles (Fig 7) and fusion of some of these vesicles with the surface membrane was observed (Fig 7a). Moreover, labeling of albumin localized on the plasma membrane (Fig 7, a and b) was significantly higher in incubations with G-CSF and FMLP than in the controls.

**DISCUSSION**

We examined the immediate effects of in vivo administration of G-CSF on circulating neutrophils. We found that G-CSF induces in vivo activation of neutrophils, as shown by the mobilization of secretory vesicles (LAP, CD11b) as well as specific granules (lactoferrin, CD11b, and CD66b) and azurophil granules (elastase α1AT).

**Mobilization of secretory vesicles.** Fusion of secretory vesicles with the plasma membrane was already observed at 30 minutes postinjection, as reflected by a decrease in intracellular alkaline phosphatase content of the neutrophils and by an increase in membrane-bound CD11b. We confirmed by immuno EM that secretory vesicles mobilize after G-CSF treatment in vitro (Table 1). Using alkaline phosphatase as a marker in immuno EM studies, Kobayashi and Robinson described in thick sections an intracellular neutrophil compartment that, after activation with FMLP, fused to form a few elongated tubular structures that associated with the cell membrane, resulting in cell surface reaction for alkaline phosphatase. Similarly, using ultrathin cryosections and albumin as a marker, we observed by immuno EM the formation of so-called large vesicles after both G-CSF and FMLP incubation. Some of these vesicles were in close association with the plasma membrane. We suppose that the compartment marked by albumin is the same as the compartment described by Kobayashi and Robinson. Thus, these large vesicles probably reflect the activation-dependent fusion of secretory vesicles, induced by G-CSF and FMLP.

Furthermore, we found elevated staining of albumin along the plasma membrane after G-CSF and FMLP incubation. Whether this reflects the exocytosis or an increased endocytosis of albumin, as has been described for ferritin, has to be evaluated.

**FcγRIII is present in secretory vesicles.** FcγRIII, one of the Fc receptors for complexed IgG, was found to be upregulated by G-CSF. FcγRIII has been found in multiple vesicles in neutrophils, and subcellular fractionation studies have suggested the presence of FcγRIII both in the specific granules and in the alkaline phosphatase-containing cell fraction. In this study, we demonstrated by immuno EM that the intracellular pool of FcγRIII is formed by secretory vesicles. The presence of FcγRIII in these easily mobilizable vesicles assures the quick replenishment of this IgG receptor in case it has been occupied or lost during phagocytosis. FcγRIII and CD11b are found in close proximity on the cell membrane, and cooperation of both molecules in signal transduction has been postulated. The presence of FcγRIII and CD11b in the same highly mobilizable intracellular compartment might facilitate an associated expression after cell activation.

Although FcγRIII, alkaline phosphatase, and CD11b are present in the same intracellular compartment, these antigens showed different kinetics after in vivo G-CSF administration. We observed in all donors secretory vesicle mobilization until 4 hours after G-CSF administration, as judged by the decrease in alkaline phosphatase content and increased CD11b expression, whereas upregulation of FcγRIII membrane-expression was found in only two out of four donors and no longer than 30 minutes postinjection (Fig 2C and 2D).

However, in vitro G-CSF incubation induced upregulation of the expression of both CD11b and FcγRIII in all tested donors, also in the two in vivo "non-responders". A similar discrepancy between upregulation of FcγRIII in vivo and in vitro has been described by Werfel et al. These investigators showed a significant upregulation of FcγRIII after C5a incubation of whole blood in vitro, whereas during hemodialysis, which leads to C5a generation, elevated FcγRIII expression on the neutrophils was found in only two out of six patients. We wondered whether this discrepancy was caused by activation-induced release of FcγRIII from the cell membrane. Indeed, in all donors we measured increasing plasma levels of sFcγRIII, accounting for the decreased membrane expression in the first hours postinjection. The level of sFcγRIII, which has a half time of 1.5 days, remained relatively constant after 4 hours postinjection. Because FcγRIII expression increases with the maturation of neutrophils, the ongoing decrease in FcγRIII expression after 4 hours postinjection might be explained by the influx of storage-pool granulocytes with lower FcγRIII expression. In vitro, after exposure to G-CSF, we only observed upregulation of FcγRIII expression and no shedding of FcγRIII, neither in whole blood cultures nor by incubation of purified neutrophils. Apparently, G-CSF is a more potent activator of neutrophils in vivo than in vitro.

**Degranulation.** As to specific granule degranulation, G-CSF induced upregulation of CD11b and CD66b and rapid release of lactoferrin both in vivo and in vitro. With respect
Fig 7. Immuno EM of ultrathin cryosections of neutrophils. Localization of albumin after neutrophil activation with FMLP (1 μmol/L, a) and with G-CSF (10 ng/mL, b). (a) After stimulation with FMLP, large vesicles strongly labeled for albumin were found (thick arrows); inside one of these vesicles, smaller vesicles can be seen (thin arrows). Fusion of a vesicle with the surface membrane is shown (arrowheads) suggesting exocytosis of albumin. Inset: Higher magnification of the plasma membrane of another neutrophil showing albumin labeling (arrows). (b) After stimulation with G-CSF, albumin is present along the plasma membrane (small arrows) and in small (thin arrows) and large vesicles (thick arrow). Inset: Area of another neutrophil at higher magnification, showing large vesicles labeled with albumin. Bars indicate 200 nm.
to the number of circulating neutrophils, in vivo release of lactoferrin was most prominent during the first 2 hours after G-CSF administration. Rising levels of lactoferrin were measured simultaneously with increased expression of CD11b and CD66b on the circulating neutrophils, reflecting the release of lactoferrin by circulating neutrophils. Lactoferrin levels peaked at 4 hours postinjection, at which time peak levels of G-CSF also were measured. As late as 3 days postinjection a second transient increase in lactoferrin plasma levels was noticed, peaking at 7 days postinjection. In close analogy with previous observations on levels of sFcerIII,\(^2,3\) this second peak most likely reflects the turnover of the abundantly produced neutrophils in the tissues, ultimately resulting in elevated plasma levels of sFcerIII and lactoferrin.

In vivo, we detected the release of azurophil granules, in sharp contrast to our in vitro observations. Expression of CD63, a specific marker for fusion of azurophil granules with the cell membrane,\(^17\) was not detected on neutrophils in response to G-CSF in vivo nor in vitro. In vivo, strongly activated neutrophils may have left the circulation and therefore be underrepresented in the blood samples used for flow cytometric analysis. Several observations are in concordance with this hypothesis: (1) After G-CSF administration, the circulating neutrophils did not exhibit decreased expression of L-selectin, in contrast to in vitro observations (this report, Sperini et al,\(^8\) and Yong and Linch\(^16\)). (2) The G-CSF-induced upregulation of CD11b expression on neutrophils in vitro was twice as high as in vivo. (3) Increased levels of CD11b on the circulating neutrophils were observed only up to 2 hours postinjection, whereas neutrophil activation in terms of granule content release was observed as late as 4 to 8 hours after administration. We propose that in vivo elastase release by G-CSF--activated neutrophils is induced by additional stimulation, which could be caused by interactions in the endothelial microenvironment. This might explain the pattern of elastase release, which is delayed and prolonged when compared with lactoferrin. The relatively prolonged elevation of elastase-\(\alpha\)1AT levels cannot be explained by a difference in half times between elastase-\(\alpha\)1AT and lactoferrin (both 45 to 60 minutes\(^{40,41}\)). Migration of neutrophils over monolayers of endothelial cells in vitro induced release of specific granules and not azurophil granules,\(^42\) therefore, neutrophil migration to the tissues alone might not account for the observed release of elastase. However, whether exposure to G-CSF influences the mobilization of these granules during migration has to be evaluated.

Functional G-CSF receptors have been detected on monocytes.\(^14,15\) In accordance with Lindemann et al.,\(^14\) we observed a decrease in the number of circulating monocytes at 30 minutes postinjection (base line values, 0.34 ± 0.03 × 10^7\) L; 30 minutes postinjection, 0.15 ± 0.03 × 10^7\) L; \(P < .05\), whereas the numbers of circulating lymphocytes and platelets were not influenced significantly by G-CSF administration. Because we did not detect elevated plasma levels of interleukin (IL)6, IL8, or tumor necrosis factor \(\alpha\) (TNF\(\alpha\)) after G-CSF administration (data not shown), we have no indications that a possible concomitant activation of monocytes contributes to a more potent neutrophil activation in vivo. Furthermore, in vitro, simultaneous incubations of whole blood with different concentrations of G-CSF and IL8 did not result in a synergistic activating effect or release of elastase (data not shown). Nevertheless, subsequent activation of the neutrophils might occur after the margination of the neutrophils.

We have shown that G-CSF administration to healthy volunteers results in immediate and significant neutrophil degranulation. According to the literature and to our own observations, the G-CSF--induced neutrophil activation apparently does not result in obvious clinical symptoms in healthy volunteers.

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