Peripheral Blood Neutrophils in Chronically Neutropenic Patients Respond to Granulocyte-Macrophage Colony-Stimulating Factor With a Specific Increase in CR1 Expression and CR1 Transcription

By Francis D. Moore, Jr, Richard M. Jack, and Joseph H. Antin

Chronically neutropenic patients from a phase I/II protocol were studied for neutrophil (PMN) abnormalities related to therapeutic use of granulocyte-macrophage colony-stimulating factor (GM-CSF). We analyzed phenotype by flow cytometry to measure indirect immunofluorescent staining and activation of transcription by in situ hybridization. PMN count increased in seven of 17 patients. For the group, PMN expression of complement receptors, CR1 and CR3, increased after GM-CSF administration ($P < .005$), while expression of class 1 and FcR III was stable. PMN from both of the patients studied by in situ hybridization demonstrated increased expression of CR1 transcript, which in one case coincided in time and intensity with the course of increased CR1 expression, while in the second case the presence of CR1 mRNA increased but lagged behind the increased CR1 protein expression. Thus, PMN activation was observed after GM-CSF infusion, as indicated by increased complement receptor expression. This effect was due both to translocation of receptors from a preformed intracellular pool to the cell surface, and to transcriptional regulation leading to increased receptor synthesis.

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T he response of the intact organism to cytokines administered for therapy has been unpredictable and complex, due to diverse modulatory effects on different end organs and due to empiric dosing routes and schedules. The cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) was introduced clinically to increase the circulating neutrophil (PMN) pool in neutropenic patients with bone marrow failure or chemotherapy. The goal of raising PMN counts was to decrease the infectious morbidity associated with neutropenia. Several series document clinical success for the goal of increasing PMN counts with GM-CSF therapy, and also point out the mild toxicities of eosinophilia, bone pain, and mild hyperpyrexia. Data on the more important end point of infection prevention are equivocal. One possibility is that the lack of salutary effect on infection might be due to abnormalities of the GM-CSF-elicited PMN.

One approach to defining the clinical effects of GM-CSF is to examine PMN function in vitro. However, this approach is limited in neutropenic patients by the low number of PMN available for pretherapy assessment. Also, the purification methods required for functional assessments can induce an inflammatory phenotype. Such technical difficulties are not encountered with either flow cytometric-based analysis or with morphologic techniques, neither of which require extensive neutrophil purification. We have examined the effect of GM-CSF administration on cell-surface phenotype and protein synthesis of PMN from neutropenic patients.

MATERIALS AND METHODS

Study design. Seventeen neutropenic patients with myelodysplastic syndrome (13 patients), aplastic anemia (three patients), or agranulocytosis (one patient) were studied. Recombinant human GM-CSF (5 $\times$ 10$^6$ colony-forming units [CFU]/mg of protein) (Immunex, Seattle, WA) was administered as a portion of a phase I/II protocol, which was approved by the Committee for Protection of Human Subjects of the Brigham and Women's Hospital. Blood was collected before GM-CSF administration, and at 2-day intervals following.

Neutrophil phenotype determinations. Chilled buffy coat leukocytes were assessed by indirect immunofluorescence and flow cytometry, as previously described. Ye-1, specific for CR1 (CD35); OKM-1 (Ortho Diagnostics, Raritan, NJ), specific for the $\alpha$-chain of CR3 (CD11b); 3G8.10, specific for FcR III (CD16); and W6/32, specific for MHC class I, were used at predetermined saturating doses. Controls for nonspecific binding used murine monoclonal antibodies of the same antibody isotype, but with irrelevant antigen specificity. Gates for granulocytes were defined by forward- and right-angle laser light scatter. Results are expressed in relative fluorescent units compared with a standard fluorescent particle.

In situ hybridization in PMN. Granulocytes were cytocentrifuged onto glass slides and fixed in freshly made, cold, buffered 4% paraformaldehyde followed by cold, 70% ethanol. purified cDNA probes specific for class I, CR1, and pUC (the cloning vector as a control) were radiolabeled to a specific activity of 2 $\times$ 10$^6$ cpm/µg. Five $\times$ 10$^5$ cpm of probe was hybridized with each slide in 0.5 $\times$ SSC (1 $\times$ SSC = 0.15 mol/L NaCl + 0.015 mol/L sodium citrate) overnight after 2 hours prehybridization. After washing twice in 2 $\times$ SSC at 25°C and once in 1 $\times$ SSC at 42°C, the slides were dipped in NTB-2 emulsion (Kodak, Rochester, NY), dried, exposed at 4°C for 6 to 10 days, and developed. As a control to assure that hybridization was occurring under the reaction conditions, PMN from normals were hybridized with actin cDNA.

Data analysis. Data sets were compared using the Mann-Whitney U test for nonparametric data.

RESULTS

Effect of GM-CSF on peripheral blood PMN counts. The clinical response of these patients has been previously reported. Circulating PMN counts increased in seven of 17 patients from 484 ± 123 cells/µL (mean ± SEM) before GM-CSF to 3,010 ± 608 cells/µL ($P < .01$).

Effect of GM-CSF on PMN cell surface expression of CR1, CR3, class I, and FcR III. For the group as a whole, blood...
granulocyte activation was observed, as manifested by increased cell surface expression of CR1 and CR3. Mean CR1 expression increased 48% from 429 relative fluorescent units before GM-CSF to 633 (Table 1). Mean CR1 expression on PMN from normal individuals was one third of that for the mean of patients before treatment with GM-CSF. Mean CR3 expression increased 59% from 378 to 599. Mean CR3 expression on PMN from normal individuals was only 27% of that seen in PMN from patients before therapy. Thus, based on the increased baseline complement receptor expression, PMN in these neutropenic patients appeared to be activated before therapy and became more activated as a result of a GM-CSF.

In contrast to the increases in complement receptor expression, levels of FcR III and class 1 remained relatively constant. Mean FcR III expression decreased slightly, from 251 before GM-CSF to 199 after GM-CSF. Mean class 1 membrane expression increased slightly from 356 to 393. Thus, we observed a specific PMN phenotypic change in response to GM-CSF, as opposed to a nonspecific change resulting in a globally increased expression of all surface antigens.

In patients who responded by increasing PMN count, CR1 expression (mean ± SD) increased 44% from 418 ± 142 relative fluorescent units before GM-CSF to 602 ± 129 after GM-CSF (P < .05, pre v post). In nonresponders, CR1 expression increased from 452 ± 217 to 630 ± 62 (P < .05, pre v post). Likewise, for responders, CR3 expression increased from 373 ± 124 to 639 ± 122 (P < .001, pre v post), while in nonresponders, CR3 expression increased from 394 ± 309 to 517 ± 235 (P = .2, pre v post). Thus, the response of the complement receptors was independent of the response of the PMN number.

Effect of GM-CSF on levels of CR1 and class 1 mRNA in PMN. PMN isolated during the course of treatment with GM-CSF were analyzed by in situ hybridization with probes specific for CR1 and class 1, assaying for relative changes in transcript levels. Cytospins were prepared for in situ hybridization on five patients. The degree of PMN morphologic preservation and the adequacy of PMN number on each slide was sufficient to allow for analysis over time in two of these five patients. Patient A responded to GM-CSF with an increase in PMN count and an increase in complement receptor cell surface expression evident by the fourth day (Table 2). In this patient, class 1 cell surface expression was also elevated on days 4 to 10 post-GM-CSF. By day 4, grains specific for CR1 transcript were detected in PMN, which increased throughout the therapy (Fig 1C and D) in parallel to the increased CR1 expression. By the end of therapy, large numbers of eosinophils had entered the circulation, which displayed strong CR1 hybridization (Fig 1F). PMN at this time also had many grains per cell of CR1-specific hybridization (Fig 1E). Grains specific for hybridization to the class 1 probe were also visible in PMN during the period of increased class 1 expression.

In patient B, the PMN count increased promptly after treatment, as did expression of CR1 and CR3 (Table 2). In situ detection of CR1 mRNA in PMN became apparent by the end of therapy. In this patient, class 1 remained constant and, likewise, there was no detectable class 1 mRNA on the autoradiographs. Thus, the increased expression of complement receptors as detected by indirect immunofluorescence was reflected by increased CR1 mRNA in peripheral blood PMN. The membrane expression of class 1, in contrast, was increased in one patient and was associated with increased transcription, while the other patient displayed constant class 1 expression and no detectable increase in class 1 transcript levels, as assessed by in situ hybridization.

**DISCUSSION**

The administration of GM-CSF to a small group of chronically neutropenic patients produced both an increase in circulating PMN numbers and an increase in PMN complement receptor expression, indicative of a change in PMN phenotype. The increase in circulating PMN number in response to GM-CSF has been previously reported for this group of patients and for others. Coincident with increasing PMN counts in response to GM-CSF infusion, mean cell surface expression of the complement receptors CR1 and CR3 increased. PMN from treated patients increased approximately 50% in complement receptor expression compared with pretreatment PMN (Table 1). As cell surface expression of CR1 and CR3 on PMN similarly increases in response to such known activators as C5a, FMLP, phorbol myristate acetate (PMA), endotoxin, tumor necrosis factor (TNF), and platelet-derived growth factor (PDGF), this increase in expression indicated that GM-CSF had caused systemic PMN activation. The observed increase in complement receptor expression in GM-CSF patients was specific and selective in that mean expression of FcR III and class 1 antigen remained constant in response to the cytokine (Table 1). The PMN appeared to be activated before therapy, with an increased CR1 and CR3 expression relative to normals. This suggested that PMN of patients were stimulated as part of the underlying disease process, perhaps due to endogenous regulatory cytokines. The phenotype-altering effect of GM-CSF was independent of its effect on the bone marrow PMN mobilization, as PMN CR1 and CR3 expression was increased to the same degree in patients who sustained an increase in PMN count and in those who did not. Thus, GM-CSF, administered in vivo, led to a specific increase in PMN cell surface expression of the complement receptors, CR1 and

Table 1. Relative PMN Surface Expression of Complement Receptors Type 1 and Type 3, FcR III, and Class 1 Proteins in GM-CSF–Treated Neutropenic Patients, as Assessed by Indirect Immunofluorescence

<table>
<thead>
<tr>
<th></th>
<th>Mean Channel Fluorescence ± SD</th>
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<tr>
<td></td>
<td>CR1</td>
</tr>
<tr>
<td>Nominals</td>
<td>138 ± 64</td>
</tr>
<tr>
<td>(n)</td>
<td>(108)</td>
</tr>
<tr>
<td>17 Patients</td>
<td>429 ± 195*</td>
</tr>
<tr>
<td>(n)</td>
<td>(18)</td>
</tr>
<tr>
<td>Before GM-CSF</td>
<td>633 ± 141†</td>
</tr>
<tr>
<td>(n)</td>
<td>(36)</td>
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Abbreviations: n, number of samples; nd, not determined.

*P < .05 compared with normals.
†P < .005 compared with before GM-CSF.
CR3, over and above that which had been induced by endogenous factors in neutropenic patients.

Increase in PMN complement receptor expression can result from increased protein synthesis and membrane

<table>
<thead>
<tr>
<th>Day of GM-CSF Therapy</th>
<th>CR1-Dependent Fluorescence</th>
<th>CR3-Dependent Fluorescence</th>
<th>Class 1-Dependent Fluorescence</th>
<th>CR1 mRNA</th>
<th>Class 1 mRNA</th>
<th>PMN (cells/μL)</th>
</tr>
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<tbody>
<tr>
<td>Patient A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>360</td>
<td>140</td>
<td>167</td>
<td>–</td>
<td>–</td>
<td>360</td>
</tr>
<tr>
<td>Day 4</td>
<td>655</td>
<td>772</td>
<td>336</td>
<td>+</td>
<td>+</td>
<td>4,200</td>
</tr>
<tr>
<td>Day 7</td>
<td>413</td>
<td>582</td>
<td>257</td>
<td>+</td>
<td>+/−</td>
<td>2,750</td>
</tr>
<tr>
<td>Day 10</td>
<td>730</td>
<td>611</td>
<td>316</td>
<td>++</td>
<td>+</td>
<td>5,460</td>
</tr>
<tr>
<td>Day 15</td>
<td>876</td>
<td>473</td>
<td>158</td>
<td>+++</td>
<td>–</td>
<td>8,775</td>
</tr>
<tr>
<td>Patient B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre</td>
<td>161</td>
<td>356</td>
<td>180</td>
<td>–</td>
<td>–</td>
<td>198</td>
</tr>
<tr>
<td>Day 2</td>
<td>542</td>
<td>723</td>
<td>180</td>
<td>–</td>
<td>–</td>
<td>580</td>
</tr>
<tr>
<td>Day 7</td>
<td>688</td>
<td>664</td>
<td>196</td>
<td>–</td>
<td>–</td>
<td>n/a</td>
</tr>
<tr>
<td>Day 12</td>
<td>411</td>
<td>385</td>
<td>142</td>
<td>+++</td>
<td>–</td>
<td>896</td>
</tr>
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Abbreviation: n/a, not available.

Fig 1. PMN preparations from a GM-CSF–treated neutropenic patient (patient A, Table 1) hybridized with radiolabeled CR1 cDNA in situ, counterstained, and exposed to photographic emulsion. (A) Cells from treatment day 15 hybridized to pUC cDNA. There are no grains visible in this control for nonspecific hybridization using cDNA from the cloning vector alone. (B) Pretreatment cells hybridized to CR1 cDNA. There are no grains visible, indicating no detectable CR1 mRNA. (C) Cells from treatment day 4 hybridized to CR1 cDNA. All cells are associated with grains, indicating presence of CR1 mRNA. (D) Cells from treatment day 7 hybridized to CR1 cDNA. There are more grains per cell than in (C), indicating higher levels of CR1 mRNA. (E) Cells from treatment day 15 hybridized to CR1 cDNA. The number of grains per cell has increased further. Cells shown are PMN. (F) Cells from treatment day 15 hybridized to CR1 cDNA. This is a second microscopic field from the same slide as (E). This shows many eosinophils, all associated with a large number of grains and indicating eosinophil CR1 transcriptional activity.

insertion of nascent complement receptors induced by GM-CSF, as previously shown in vitro. This increase in translation is likely a function of increased levels of complement receptor transcript. Since PMN numbers in these neutropenic patients were too small for RNA blot analysis or protein biosynthetic experiments, in situ hybridization was used to determine whether increased levels of CR1 transcript were associated with the increased levels of CR1 expression (Fig 1). This approach had the additional advantage of allowing direct visualization of those cell types that were responding to GM-CSF. As shown in Table 2, increased receptor expression correlated in time with increased hybridization to CR1-specific cDNA. For patient A in Fig 1, the predominant cell type demonstrating increased in situ hybridization was the neutrophil, confirming the flow cytometer–derived data that PMN were affected. Eosinophils became numerous by the end of GM-CSF therapy in this patient and also demonstrated in situ hybridization to the CR1 probe. It was possible that, late in therapy, the PMN phenotype data became corrupted by migration of complement receptor–expressing eosinophils into the PMN analysis gates of the flow cytometer. However, given the obvious increased in situ hybridization to the CR1 probe by the PMN, the predominant granulocyte of the in situ samples and differential counts during the course of therapy, the phenotype data were unlikely to have been misleading. Patient A also demonstrated a level of class 1 mRNA and surface expression that increased and decreased in tandem, further strengthening the link between the phenotypic and in situ data. A previous report would indicate that class 1 expression is not responsive to GM-CSF, as would the data for our entire patient group. Based on patient A, one would postulate that one mechanism by which PMN expression of cell surface receptor responded to GM-CSF was by increased transcription, leading to increased synthesis of receptor.

In patient B of Table 2, CR1 expression increased and was not accompanied by a detectable increase in CR1 mRNA until late in the course of therapy. This indicated that this patient responded with increased expression initially by translocation of receptor from a preformed intracellular pool to the cell surface, and only then supple-
mented by increased transcript with subsequent CR1 translation and expression. Upregulation of PMN CR1 from intracellular storage sites stimulated by GM-CSF is similar to the cellular activation of PMN by C5a,4 FMLP,4 endotoxin,13 and TNF.4 This mechanism may have also explained the pretherapy elevations of CR1 expression in the absence of detectable CR1 mRNA. In patient B, expression of class 1 was unaffected and its mRNA was undetected. Thus, GM-CSF may increase expression of CR1 in vivo by two mechanisms: by increased synthesis and by movement of already synthesized CR1 to the cell surface from the interior of the cell. This may also be the first demonstration of transcriptional regulation in mature PMN in vivo.

These data suggest that PMN from GM-CSF–treated patients are activated, as assessed by their increased expression of CR1 and CR3 both before GM-CSF infusion and subsequent to treatment. Whether the patients’ PMN are intrinsically abnormal or whether the PMN are normal cells in an abnormal milieu has not been determined. The translocation of preformed CR1 to the cell surface is a characteristic of PMN activation and cells exhibiting this type of activation have exhibited deficient chemotaxis in settings of thermal injury and endotoxemia.14 Indeed, after GM-CSF treatment, PMN chemotaxis into skin windows becomes deficient19 in autologous bone marrow transplant patients, and in vitro chemotaxis of PMN from patients with normal PMN counts is reduced.20 Chemotaxis of normal PMN exposed to GM-CSF in vitro is also impaired.21 Increased synthesis of protein has been equated to “priming” and may render the cells hyperfunctional, in analogy to phenomena seen with macrophages. Such priming has been reported for in vitro exposure of normal PMN to GM-CSF.22,23 In this experiment, there were insufficient numbers of PMN pretherapy to allow for purification and in vitro functional assessments. But, given that increased receptor expression and its new synthesis was manifest in response to GM-CSF, the GM-CSF–elicited PMN may not be normally functional, in that it is activated in an inappropriate location at an inappropriate time. The physiologic influence of GM-CSF on PMN is expressed at the local level and maintains competent cells at the inflammatory locus by promoting synthesis of necessary receptors and by impairing further chemotaxis. It is the systemic exposure to large amounts of GM-CSF that is nonphysiologic. Such observations raise the possibility that GM-CSF treatments, designed to improve PMN function by increasing PMN concentrations, instead impair PMN function.

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Peripheral blood neutrophils in chronically neutropenic patients respond to granulocyte-macrophage colony-stimulating factor with a specific increase in CR1 expression and CR1 transcription

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