Effects of Granulocyte Colony-Stimulating Factor on Plasma Cytokine and Cytokine Receptor Levels and on the In Vivo Host Response to Endotoxin in Healthy Men

By Thomas Pollmacher, Carsten Korth, Janet Mullington, Wolfgang Schreiber, Joachim Sauer, Helmut Vedder, Chris Galanos, and Florian Holsboer

We investigated the effects of granulocyte colony-stimulating factor (G-CSF) on cytokine and cytokine receptor plasma levels and on the in vivo host response to Salmonella abortus equi endotoxin in healthy males. Twenty volunteers received 0.8 ng/kg endotoxin and saline intravenously 1 week apart in randomized order. Twelve hours before both experiments, 10 of these subjects were pretreated with 300 µg G-CSF subcutaneously. G-CSF itself increased granulocyte and monocyte counts and the plasma levels of tumor necrosis factor-α (TNF-α), soluble TNF receptors (sTNF-R) p55, and p75 and interleukin-1 receptor antagonist (IL-1ra). G-CSF did not influence plasma IL-1β and IL-6 levels. In the G-CSF-pretreated subjects endotoxin-induced surges in rectal temperature and in the plasma levels of TNF-α plasma levels were about 50% increased, and surges in the plasma levels of both sTNF-Rs and IL-1ra were about twice as high as in the control group. Endotoxin-induced increases in IL-6, cortisol, and heart rate were not modified by G-CSF pretreatment. Endotoxin administration induced a transient 50% reduction in leukocyte counts in the G-CSF-pretreated subjects that was not seen in the control group. We conclude that a single standard dose of G-CSF increases the plasma levels of cytokines and cytokine receptors and considerably modifies the host response of healthy humans to a low dose of endotoxin.

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Materials and Methods

Subjects. Twenty healthy male volunteers took part in the present investigation after written informed consent had been obtained. The experimental protocol had received ethical approval. Before enrollment in the study, all potential participants were screened with a medical history, physical examination, laboratory investigations, electrocardiogram (EKG), and electroencephalogram to exclude individuals with acute or chronic disease.

Experimental procedures. In all subjects, the effects of 0.8 ng/kg body weight of Salmonella abortus equi endotoxin on the primary host response were evaluated in a single-blind design. Endotoxin was available as a protein-free sterile solution; details of the preparation have been reported earlier. In randomized order, endotoxin and 0.9% saline solution were given 1 week apart as a bolus intravenous injection at 0900 hours. Twelve hours before both the administration of endotoxin and placebo, 10 subjects were pretreated with 300 µg of human recombinant G-CSF (Neupogen, purchased from Hoffmann LaRoche Ltd, Grenzach, Germany). All G-CSF vials used were from one batch. Contamination with endotoxin was excluded in one vial of this batch by the Limulus-lysate assay.

Throughout every experimental session, one-lead EKG and rectal temperature (temperature monitor model 8055, S&W, Albertslund, Denmark) were continuously monitored. Blood was drawn through an indwelling venous catheter. Blood samples were stabilized with Na-EDTA (1 mg/mL) and aprotonin (300 KIU/mL), centrifuged and aliquoted, and plasma was frozen immediately to −20°C (for details see reference 5). Blood pressure was monitored with a Dinamap Vital.

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Daten Monitor 1846SX (Critikon, Norderstedt, Germany). Subjects remained in bed and were under continuous observation for 12 hours before and 24 hours following the administration of endotoxin and placebo, respectively. An experienced physician was on call during the whole experimental procedure. Emergency equipment was always available.

Assays. Blood cell counts were determined with a Coulter counter ST3 (Coulter Inc, Krefeld, Germany). IL-1α, TNF-α, sTNF-R p55, sTNF-R p75, and IL-6 were determined by enzyme-linked immunosorbent assays (Medigene Diagnostics, Brussels, Belgium). For all these assays, the interassay and intraassay coefficients of variation were below 5% and 8%, respectively. Detection limits were 2 pg/mL for IL-1α, 3 pg/mL for TNF-α and IL-6, 50 pg/mL for sTNF-R p55, and 100 pg/mL for sTNF-R p75. To optimize the sensitivity of the TNF-α assay, we added an additional standard (4.3 pg/mL). The correlation of 24 consecutively sampled duplicate measurements in the range below 13 pg/mL was r = 0.99 (Pearson’s correlation; P < .01), demonstrating that in our hands the assay performs well, even in this low range of values. IL-1α was measured by an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN). The interassay and intraassay coefficients of variation were below 5%. The detection limit was 22 pg/mL. Plasma cortisol concentrations were measured by a coated tube radioimmunooassay (ICN Biomedicals, Carson, CA). Interassay and intraassay coefficients of variation were below 7%. The detection limit was 1.5 ng/mL. For all assays, all samples of a particular subject were processed in the same kit.

Statistics. Data were processed with commercially available statistical software (SPSSPC+). Differences in the time courses of host response parameters were assessed by analysis of variance for repeated measures. Simple main effects were tested with unpaired Student’s t-tests. As the major response to a low dose of endotoxin was heart rate, no musculoskeletal pain was reported after G-CSF administration. G-CSF did not influence serum alkaline phosphatase, gamma-glutamyl transferase, lactate dehydrogenase, or uric acid levels as assessed 36 hours after injection.

RESULTS

Effects of G-CSF alone. To evaluate the influence of G-CSF alone on indices of the host response, we compared the time courses of the respective parameters between G-CSF-treated and control subjects during those experimental sessions when the volunteers received placebo at 900 hours (Table 1, Fig 1).

The controls and the G-CSF-pretreated subjects did not differ with respect to age (25.8 ± 0.8 v 26.9 ± 1.4 years). Baseline (2100 hours) leukocyte (5.7 ± 0.3 v 6.5 ± 0.3 x 10⁹/L), neutrophil (3.2 ± 0.3 v 3.8 ± 0.3 x 10⁹/L), monocyte (0.4 ± 0.1 v 0.3 ± 0.1 x 10⁹/L), and lymphocyte counts (2.1 ± 0.1 v 2.4 ± 0.2 x 10⁹/L) did not differ between the control and the G-CSF-treated groups. G-CSF induced large increases in the leukocyte and neutrophil counts, persisting for 24 hours postinjection. Monocyte counts were also increased by G-CSF, whereas lymphocyte counts were not influenced at all.

Baseline plasma IL-1ra (132 ± 21 v 194 ± 75 pg/mL), TNF-α (3.8 ± 1.2 ± 2.1 ± 0.7 pg/mL), sTNF-R p55 (1.4 ± 0.1 v 1.4 ± 0.1 ng/mL), sTNF-R p75 (2.9 ± 0.4 v 3.1 ± 0.2 ng/mL), and heart rate (61 ± 3 v 62 ± 3 beats/min) did not differ significantly between the control group and the G-CSF-treated subjects. G-CSF increased the plasma levels of IL-1ra to about 0.6 ng/mL (F[1,18] = 6.7; P < .05), TNF-α to about 8 pg/mL (F[1,18] = 5.0; P < .05), sTNF-R p55 to about 2 ng/mL (F[1,18] = 6.7; P < .05), and sTNF-R p75 to about 5 ng/mL (F[1,18] = 11.9; P < .01), whereas in the control group, the respective plasma levels remained constant throughout the experimental time period. In addition, heart rate did not decrease during the night following G-CSF administration in contrast to the control group (F[1,18] = 7.7; P < .05). All these effects described were sustained for 24 hours after G-CSF injection. Plasma IL-6 (F[1,18] = 1.85; not significant [NS]), cortisol (F[1,18] = 2.06; NS), temperature (F[1,18] = 2.27; NS), systolic (F[1,18] = 0.02; NS), and diastolic blood pressure (F[1,18] = 0.56; NS) were not significantly influenced by G-CSF. IL-1β plasma levels slightly above the detection limit could be detected in only four subjects of the control group and in two G-CSF–treated subjects. Therefore, analysis of variance (ANOVA) was not performed.

None of the subjects reported any side effects; in particular, no musculoskeletal pain was reported after G-CSF administration. G-CSF did not influence serum alkaline phosphatase, gamma-glutamyl transferase, lactate dehydrogenase, or uric acid levels as assessed 36 hours after injection.

Influence of G-CSF on endotoxin-induced host response. Immediately before endotoxin administration, rectal temperature (36.5 ± 0.1 v 36.6 ± 0.1°C), plasma IL-1β (4.6 ± 4.5 v 0.4 ± 0.3 pg/mL), IL-6 (7.6 ± 5.5 v 6.2 ± 3.5 pg/mL), and cortisol (135 ± 17 v 129 ± 12 ng/mL) levels were similar in the controls and in the G-CSF–treated subjects, respectively. Consistent with the effects of G-CSF outlined above, G-CSF–treated subjects again showed higher IL-1ra (0.13 ± 0.02 v 1.10 ± 0.44 ng/mL, P < .05), TNF-α

Table 1. White Blood Cells 12 and 24 Hours After 300 µg G-CSF Subcutaneously Compared With Control Subjects

<table>
<thead>
<tr>
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<th>+12 h</th>
<th>+24 h</th>
<th>ANOVA for Repeated Measures Condition Effect</th>
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</thead>
<tbody>
<tr>
<td>Leukocytes (x10⁹/L)</td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>5.2 ± 0.5</td>
<td>6.1 ± 0.3</td>
<td>F(1,18) = 102.7, P &lt; .01</td>
</tr>
<tr>
<td>G-CSF</td>
<td>27.1 ± 2.5</td>
<td>24.6 ± 1.6</td>
<td></td>
</tr>
<tr>
<td>Neutrophils (x10⁹/L)</td>
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<tr>
<td>Control</td>
<td>3.2 ± 0.4</td>
<td>3.3 ± 0.3</td>
<td>F(1,18) = 93.34, P &lt; .01</td>
</tr>
<tr>
<td>G-CSF</td>
<td>24.3 ± 2.3</td>
<td>21.6 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Monocytes (x10⁹/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.2 ± 0.0</td>
<td>0.4 ± 0.1</td>
<td>F(1,12) = 5.9, P &lt; .05</td>
</tr>
<tr>
<td>G-CSF</td>
<td>0.9 ± 0.2</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (x10⁹/L)</td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>1.7 ± 0.1</td>
<td>2.2 ± 0.2</td>
<td>NS</td>
</tr>
<tr>
<td>G-CSF</td>
<td>1.9 ± 0.1</td>
<td>2.7 ± 0.2</td>
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</table>

Abbreviation: NS, not significant.
In the present investigation, a standard dose of G-CSF, in addition to its known enhancing effects on granulocyte and monocyte counts, increased plasma cytokine and cytokine receptor levels in healthy subjects. Furthermore, certain time by condition interaction effects for endotoxin-induced changes in leukocyte counts (F[2,36] = 64.4; P < .01), temperature (F[4,72] = 2.98; P < .05), TNF-α (F[4,72] = 5.81; P < .01), sTNF-R p55 (F[4,72] = 12.8; P < .01), sTNF-R p75 (F[4,72] = 12.9; P < .01), IL-1ra (F[2,36] = 8.11; P < .01), and IL-6 (F[2,36] = 3.62; P < .05). Whereas in the control group endotoxin induced an increase in the leukocyte count without prior transient leukopenia, G-CSF-pretreated subjects showed a rapid and substantial drop to about 50%, which was followed by an increase, but not to the preendotoxin level. As compared with the controls, the G-CSF-pretreated subjects showed higher endotoxin-induced increases in rectal temperature (2 and 3 hours postinjection), in TNF-α (1 and 2 hours postinjection), sTNF-R p55 (1 to 5 hours postinjection), sTNF-R p75 (1 to 5 hours postinjection), and in IL-1ra (3 hours postinjection). Despite the significant time by condition interaction, IL-6 levels did not differ between the groups significantly at any of the time points tested. Endotoxin treatment increased heart rate (F[4,72] = 8.8; P < .01) and plasma cortisol levels (F[4,72] = 3.73; P < .01). However, neither heart rate (F[4,72] = 1.59; NS) nor cortisol increases (F[4,72] = 2.15; NS) were influenced by G-CSF pretreatment. Endotoxin did not significantly influence systolic (F[4,72] = 1.26; NS) or diastolic (F[4,72] = 2.03; NS) blood pressure. Neither the G-CSF pretreated nor the control subjects showed detectable increases in the plasma IL-1β level after endotoxin. The frequency of endotoxin-induced side effects did not differ between the groups (short lasting: chills; three v three subjects, headache: three v five subjects, and body aches: three v one subject in the control group and the G-CSF-pretreated groups, respectively).

**DISCUSSION**

In the present investigation, a standard dose of G-CSF, in addition to its known enhancing effects on granulocyte and monocyte counts, increased plasma cytokine and cytokine receptor levels in healthy subjects. Furthermore, certain in vivo host responses to endotoxin administered 12 hours after G-CSF were augmented.

**Effects of G-CSF alone.** G-CSF treatment increased the plasma levels of TNF-α, sTNF-R p55, sTNF-R p75, and IL-1ra. G-CSF did not influence rectal temperature, plasma IL-1β, IL-6, and cortisol levels, but it did prevent the well-known circadian drop in heart rate. G-CSF induced subtle decreases in the plasma levels of TNF-α and IL-1ra that were one to two orders of magnitude smaller than those observed in response to endotoxin, whereas the G-CSF–induced increases in the plasma levels of sTNF-R p55 and p75 reached about one and two thirds of the respective increases induced by endotoxin in the control group (compare Figs 1 and 2).
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It is very unlikely that these effects were caused by a contamination of the G-CSF preparation with endotoxin, because endotoxin was not detected by the Limulus-lysate assay.

Increased plasma cytokine and cytokine receptor levels after G-CSF treatment may be due to a release from monocytes or granulocytes that are also able to produce TNF-α, sTNF-Rs, and IL-1ra. A study by Re et al28 that shows increased IL-1ra production of neutrophils after in vitro stimulation with G-CSF lends support to the idea of a direct treatment effect related to the immediate activation of neutrophils by G-CSF. However, Re et al28 also found IL-1ra transcripts in unstimulated neutrophils. In addition, spontaneous release of sTNF-R p55 and sTNF-R p75 from neutrophils was documented by Lantz et al.30 Therefore, G-CSF-induced increases in the plasma levels of cytokines and cytokine receptors may also be caused by spontaneous release from an increased number of either neutrophils or monocytes, or both.

Effects of G-CSF on the host response to endotoxin. In the present study, endotoxin administration to healthy subjects induced the well-known pattern of host response that includes increases in the plasma levels of cytokines, cytokine receptors and cortisol, rectal temperature, and heart rate. This response pattern was considerably modified by G-CSF pretreatment. G-CSF-pretreated subjects showed larger endotoxin-induced surges in the plasma levels of TNF-α, sTNF-R p55, sTNF-R p75, and IL-1ra. Endotoxin-induced increases in plasma IL-6, cortisol, and heart rate were not changed. The increased endotoxin-induced release of cytokines and cytokine receptors after G-CSF pretreatment may be related to the increased number of monocytes and granulocytes in the circulation. Increased sensitivity of neutrophils to endotoxin may also be involved, because it has recently been reported that G-CSF treatment upregulates the expression of CD14 on human neutrophils.31 CD14 is one of the receptors for endotoxin complexed to endotoxin-binding protein,32 and transgenic mice expressing human CD14 are hypersensitive to endotoxin.33

Our finding of an increased temperature response to endotoxin following G-CSF treatment could be due to the increased amounts of circulating TNF-α, known to be pyrogenic. The assay we used measures total TNF-α that includes TNF bound to sTNF-Rs. Based on in vitro studies it has been argued that the latter may not be biologically active. Because G-CSF alone increased the plasma levels of both sTNF-Rs, and the subsequent endotoxin-induced increase also was considerably augmented, one might expect to see a decreased biological activity of TNF-α. However, the modulation of TNF-α bioactivity by its own soluble receptors in vivo is complex. Soluble TNF-R p55 attenuated TNF-α bioactivity in nonhuman primates, but sTNF-R p75 increased bioactivity in mice. Furthermore, it has been suggested that both sTNF-Rs stabilize the most active, trimeric form of TNF-α. Therefore, although our clinical investigation does not allow a definite conclusion, our results support the assumption that the increased endotoxin-induced temperature response after G-CSF treatment is caused by an increased amount of biologically active TNF-α in the circulation.

The results we present here are in line with a study by...
Hartung et al\textsuperscript{11} that reports increased ex vivo release of IL-1\textalpha and sTNF-Rs from whole blood that was stimulated with endotoxin after G-CSF treatment in healthy volunteers. In contrast to our results, this study found no effect of G-CSF on endotoxin-induced TNF-\textalpha protein release and bioactivity. This discrepancy is probably due to the different experimental settings. Ex vivo stimulation does not stimulate the host response of the entire organism. In addition, low-dose endotoxemia induces a moderate and self-limited host response, whereas ex vivo models that use high amounts of endotoxin assess maximal cytokine release capacity. A study that investigated in parallel the endotoxin-induced in vivo host response and ex vivo cytokine release illustrates how different the results of the two experimental approaches can be. Cancer patients who were treated daily with \textit{Salmonella abortus equi} endotoxin showed a progressive decline in the peak serum cytokine levels, including TNF-\textalpha and IL-6. In contrast, ex vivo stimulated mononuclear cells that were prepared from peripheral blood drawn before each endotoxin treatment showed a progressive increase in the release capacity for the same cytokines.\textsuperscript{30}

In the subjects without G-CSF pretreatment, the low dose of endotoxin we used did not induce the transient leukopenia reported in studies using \textit{Escherichia coli} endotoxin.\textsuperscript{6,34} However, injection of a higher amount of the same endotoxin preparation that we used in this study, caused a transient decline in leukocyte counts with its nadir 2 hours postinjection.\textsuperscript{40} In the present study, G-CSF-treated subjects showed a profound transient drop in leukocyte counts 2 hours after endotoxin administration, thus suggesting that G-CSF pre-treatment augments endotoxin-induced transient leukopenia. This finding is best explained by the well-known activating effects of G-CSF on neutrophils that includes its influence on adhesion molecules.\textsuperscript{29,41-47} Again, G-CSF-induced upregulation of CD14 expression\textsuperscript{31} may be involved, because CD14 mediates the increasing effect of endotoxin on the adhesive activity of CR3 (CD11b/CD18) on neutrophils.\textsuperscript{54} Neutrophil adhesion, in turn, is a poten stimulus for the shedding of sTNF-Rs from neutrophils\textsuperscript{50} and, therefore, it may contribute to the increased endotoxin-induced surges in plasma levels of sTNF-Rs in G-CSF-treated subjects.

In summary, a single standard dose of G-CSF induced increases in plasma cytokine and cytokine receptor levels and considerably modified major aspects of the host response to a low dose of endotoxin administered 12 hours after G-CSF to healthy volunteers.

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