Protective Effect of Granulocyte Colony-Stimulating Factor Against T-Cell-Meditated Lethal Shock Triggered by Superantigens

By Yoshiyasu Aoki, Kenji Hiromatsu, Noritada Kobayashi, Tomomitsu Hotta, Hidehiko Saito, Hideo Igarashi, Yoshiyuki Niho, and Yasunobu Yoshikai

The bacterial superantigens (SAg), toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxin B (SEB), are powerful T-cell stimulators, triggering systemic release of lymphokines causing lethal shock in D-galactosamine (D-Gal)-sensitized mice. We show that pretreatment with recombinant human granulocyte colony-stimulating factor (rhG-CSF) protects mice against T-cell-mediated SAg shock. In mice challenged with D-Gal/TSST-1, lethal shock was caused within 30 hours. In contrast, animals pretreated with two consecutive subcutaneous injections of 2 μg rhG-CSF with a 12-hour time interval showed only marginal signs of illness and no lethality after challenge with D-Gal/TSST-1. Mice treated with 5 μg rhG-CSF either 12 or 6 hours in advance also survived otherwise lethal doses of D-Gal/TSST-1. The protective effects of rhG-CSF pretreatment was also evident against lethal doses of D-Gal/SEB challenge and this protection was accompanied by suppression of systemic interleukin-2. However, rhG-CSF affected neither the proliferative responses of SAg-reactive T cells in vivo or in vitro nor their interleukin-2 production in vitro, implying that rhG-CSF may indirectly interfere with cytokine synthesis in T cells but not with T-cell-SAg binding itself. These results represent another beneficial effect of rhG-CSF as an anti-inflammatory agent against T-cell-mediated toxicity triggered by SAg. © 1995 by The American Society of Hematology.

It is well known that Staphylococcus aureus exotoxins are responsible for a wide range of clinical conditions, including food poisoning, scalded skin syndrome, scarlet fever, and shock. Septic shock is a systemic response to infection with high mortality. Although septic shock has typically been recognized as a consequence of gram-negative bacteraemia, up to 30% of cases are associated with gram-positive bacteria. Toxic shock syndrome (TSS) is characterized by acute onset, fever, hypotension, a scarlatiniform rash, and failure of major organ systems. TSS is caused by exotoxins such as toxic shock syndrome toxin 1 (TSST-1) usually in the setting of tampon use during menses or after surgery or trauma. These toxins are thought to exert their pathogenic effects by the massive activation of T lymphocytes and are now grouped with superantigens (SAg).

SAg have been operationally defined as bifunctional molecules that bind to major histocompatibility complex (MHC) class II structures and activate T cells expressing appropriate Vβ segments of T-cell receptors (TCR). Clonal expansion of the relevant population in vivo is followed by clonal deletion because of apoptosis in the periphery. SAg have many effects on lymphocytes. They induce the production of various T-cell lymphokines, and release of these endogenous mediators leads to a number of pathophysiologic reactions such as fever, leukopenia, disseminated intravascular coagulation, and hemodynamic changes that may ultimately lead to lethal shock.

For treatment of severe infections, recombinant human granulocyte colony-stimulating factor (rhG-CSF) represents a powerful agent that can enhance the functional activity of neutrophils. G-CSF is a glycoprotein of 174 (human G-CSF) or 178 (murine G-CSF) amino acids, and considerable similarity exists in the amino acid sequences of human and murine G-CSF and in the sequence of their receptors. The broad species cross-reactivity of human G-CSF has allowed the in vivo action of purified rhG-CSF to be studied in mice. The actions of G-CSF range from specifically stimulating neutrophil differentiation and proliferation to its general effects on mature neutrophils. In patients receiving intensive cytotoxic chemotherapy for cancer, the use of rhG-CSF has been shown to accelerate the recovery of neutrophil counts in peripheral blood and decrease the incidence of fever and infection. Administration of rhG-CSF even after the onset of sepsis has been reported to lead to improved survival in experimental animals.

In consideration of the above points, we designed the present study to test rhG-CSF as an anti-inflammatory agent. We used purified bacterial exotoxins, TSST-1 and staphylococcal enterotoxin B (SEB), instead of infecting mice with staphylococci, and examined the effects of high doses of rhG-CSF on the T-cell-mediated lethal shock triggered by SAg. Pretreatment with rhG-CSF protected mice against T-cell-mediated SAg shock accompanied by diminished interleukin-2 (IL-2) production in vivo. These results provide insight into the therapeutic use of rhG-CSF in preventing SAg-induced lethal shock.

MATERIALS AND METHODS

Mice. Female BALB/c (H-2b) mice were purchased from Japan SLC (Shizuoka, Japan). Mice were 2 to 3 months old and weighed 23 to 25 g were used in all experiments.

Reagents and injection protocol. Purified rhG-CSF was a generous gift from Kirin Brewery Co Ltd (Tokyo, Japan). rhG-CSF contained less than 1.0 endotoxin U/mg of protein. Recombinant murine...
INTERFERON-Gamma (IFN-gamma) and IL-4 were purchased from Genzyme Corp (Cambridge, MA). TSST-1 was isolated and prepared as described previously,17 SEB, lipopolysaccharide (LPS), and concanavalin A (Con A) were purchased from Sigma Chemical Co (St Louis, MO). D-Gal was purchased from Tokyo Chemical Industry Co, Ltd (Tokyo, Japan). Mice received intraperitoneal (IP) injections of either 10 µg TSST or 10 µg SEB with 20 mg D-Gal. Subcutaneous (SC) injections of rhG-CSF (0.5 to 5 µg/body) in these mice were performed at the time points indicated in the Results. Control mice received the same volume of phosphate-buffered saline (PBS).

Monoclonal antibodies (MoAbs). Anti-Vβ6 MoAb (44-22-1), kindly provided by Dr H. Hengartner (Ludwig Institute for Cancer Research, Epalinges, Switzerland), was purified and conjugated with fluorescein isothiocyanate (FITC) in our laboratory. Purified and FITC-conjugated anti-Vβ8.1/8.2 MoAbs were purchased from Pharmingen (San Diego, CA). Phycoerythrin (PE)-conjugated anti-CD4 MoAb (LJ34) was purchased from Becton Dickinson (Mountain View, CA). PE-conjugated anti-CD8 MoAb was purchased from Caltag Laboratories (San Francisco, CA). Coating and detecting MoAbs to perform ELISA were obtained from Pharmingen.

Hematology. Mice were bled from the retro-orbital plexus using heparinized capillaries, and total blood cell counts were determined using a hemocytometer. Blood smears were fixed with ethanol for May-Giemsa staining to count leukocyte differentials. The absolute numbers of neutrophils were calculated by multiplying the total white blood cell (WBC) counts by the differential counts.

Proliferation assays. Mice were injected SC with either 5 µg of rhG-CSF or with the same volume of PBS 6 hours before death. No D-Gal was used in this experiment. Whole spleen cells that had been depleted of erythrocytes with 0.16 mol/L ammonium chloride were cultured in 96-multifwell tissue culture plates (Nunc, Roskilde, Denmark) in quadruplicate at 1 x 10^6 cells/well. Cultures were stimulated with both SEA (2.5 µg/mL), TSST-1 (10 ng/mL), SEB (10 µg/mL), or Con A (2 µg/mL) in 0.1 mL RPMI1640 (GIBCO Laboratories, Grand Island. NY) containing 10% fetal calf serum (FCS), 5x10^-5 M L-glutamine, 5 x 10^-5 M L-2-ME, and antibiotics in the presence or absence of rhG-CSF at the concentrations indicated in the Results. The plates were incubated at 37°C in an atmosphere containing 5% CO2. Cultures were harvested 48 hours later after a 6-hour pulse with 1 µCi/well of [3H]-TdR (Amersham, Buckinghamshire, UK). Radioactivity was determined in a liquid scintillation beta counter.

Preparation of serum. Blood was taken from the retro-orbital plexus at the indicated time points after treatment. Samples were subsequently centrifuged at 5,000 rpm, and the supernatants were collected and stored individually at -70°C until use in the cytokine assay.

Cytokine measurement. Cytokine levels in mouse serum and cultured supernatants were evaluated using an ELISA. ELISA for tumor necrosis factor-a (TNF-a), IL-1, and IL-2 were performed using commercially available kits from Genzyme Co, IL-10 and granulocyte-macrophage colony-stimulating factor (GM-CSF) levels were determined using ELISA kits from Endogen Inc (Boston, MA). Assays were performed in duplicate exactly as described by the manufacturers. ELISAs for IFN-gamma and IL-4 were performed using Pharmingen MoAbs according to the manufacturer's instructions.

Flow cytometry analysis. For two-color flow cytometry analysis, lymph node cells and spleen cells were taken from SEB-treated mice. Mice were analyzed individually. Cells were stained sequentially with one of the FITC-labeled anti-β MoAbs mentioned above, followed by PE-anti-CD4 or PE-anti-CD8 MoAbs. Two-color analysis was performed with a FACScan Lysis II (Becton Dickinson). We carefully gated cells by forward and sideward scattering of live lymphocytes, and the expression of TCR Vβs on CD4+ or CD8+ cells was analyzed. The percentage of fluorescence-positive cells was determined by integrating profiles based on 3 x 10^4 CD4+ or CD8+ lymphocytes.

Statistical analysis. Statistical comparisons were made using the Student's t-test except for lethality data, which were analyzed using the the generalized Wilcoxon's test. A P value of less than .05 was taken to be significant.

RESULTS

G-CSF induces in vivo resistance to the lethal effect of TSST-1 plus D-Gal. Although mice are more resistant than humans to the pathogenic effects of bacterial toxins, septic shock can be induced by low doses of endotoxin or exotoxin after sensitization with D-Gal.18 One group of BALB/c mice received an IP injection of 20 mg of D-Gal and, simultaneously, 10 µg of TSST-1. In accordance with published data,1 Fig 1 shows that D-Gal/TSST-1 caused lethal shock within 30 hours. The first signs of illness, eg, immobility or rough fur, were apparent around 8 to 10 hours after the administration of D-Gal/TSST-1. Control mice treated with D-Gal or TSST-1 alone showed no lethality throughout an observation period of several days. In contrast, pretreatment with rhG-CSF led to development of resistance to lethal shock in mice challenged with D-Gal/TSST-1. Animals that were pretreated with two consecutive SC injections of 2 µg rhG-CSF with a 12-hour time interval showed similar signs of illness but no lethality after challenge with D-Gal/TSST-1. Pretreatment with 1 µg of rhG-CSF was also effective. However, further reductions in the dose of rhG-CSF resulted in achieving only minor protection, ie, 80% and 40% survival for 1 and 0.5 µg of rhG-CSF, respectively. Mice surviving 80 hours after challenge with D-Gal/TSST-1 recovered completely from illness.

We then examined whether a single SC injection of rhG-CSF was capable of leading to development of resistance to lethal shock. The majority of mice treated with 5 µg of rhG-CSF either 12 or 6 hours in advance survived otherwise lethal doses of D-Gal/TSST-1 (Fig 2). When, instead of being administered in advance, rhG-CSF was administered simultaneously with D-Gal/TSST-1 or 6 hours after, no protection was observed (Fig 2). A single SC injection of rhG-CSF is sufficient but has to be administered in advance to achieve sufficient protection against lethal shock induced by D-Gal/TSST-1.

SAg-induced neutropenia. Leukocyte counts were monitored during SAg-shock (Fig 3). Six hours after SC administration of 5 µg of rhG-CSF, mice received TSST-1 with or without D-Gal. TSST-1 injection caused a marked decrease in circulating leukocyte counts in both rhG-CSF--treated and untreated mice (Fig 3A and B). In untreated mice, absolute neutrophil counts (ANC) decreased to one third of the baseline level (Fig 3C and D). D-Gal administration itself did not affect the extent of the decrease in neutrophils. ANC were significantly increased in rhG-CSF--treated mice compared with those in untreated mice before and after challenge with D-Gal/TSST-1. However, ANC at 6 hours after D-Gal/TSST-1 challenge were preserved to the levels of naive mice (Fig 3C and D). Twelve hours later, ANC recovered to the baseline levels.

G-CSF modulates systemic cytokine release in mice after...
**TSST-1/D-Gal challenge.** SAg induce systemic release of various T-cell-dependent lymphokines that are key mediators in SAg-induced shock models. In the first approach, we investigated the circulating levels of TNF-α and IL-2. Systemic TNF-α levels in rhG-CSF-treated mice were as high as in untreated animals after TSST-1 administration (Fig 4A). In contrast, IL-2 was detected at much lower levels in G-CSF–treated animals (Fig 4B). The mean IL-2 levels in G-CSF–treated mice were 34% of those of controls at 2 hours and 48% at 6 hours after D-Gal/TSSST-1 challenge. There were no marked differences in the profiles of these cytokines between D-Gal–sensitized and –unsensitized animals (data not shown). Next, we examined the profiles of IFN-γ and IL-1α, which are known to potentiate the toxicity of TNF. Systemic IL-1 was upregulated 2 hours after TSST-1 injection (Fig 4C) and IFN-γ appeared in the circulation 6 hours after the administration of TSST-1 (Fig 4D). Pretreatment with G-CSF reduced the production of neither IL-1 nor IFN-γ in response to TSST-1 in vivo. No significant reduction of GM-CSF release was not observed either (Fig 4E). Finally, we analyzed the serum levels of anti-inflammatory cytokines, and the results showed that the release of IL-4 into the bloodstream was detected 6 hours after TSST-1 administration. As shown in Fig 4F, IL-4 serum levels varied to such a degree between samples that we could not find a significant difference in IL-4 levels between rhG-CSF–treated mice and controls. We examined serum IL-10 levels, but detectable levels of IL-10 were not released into the circulation within 6 hours of TSST-1 injection (data not shown).
**Role of G-CSF in SEB shock.** To generalize the protective effects of G-CSF against the lethal toxicity caused by bacterial SAg, mice were challenged with another exotoxin, SEB. Mice received IP injections of 20 mg of D-Gal and simultaneously of 10 μg of SEB. As reported previously, treatments with rhG-CSF (5 μg SC) 6 hours in advance and simultaneously with SEB and D-Gal. In this model, pretreatment with rhG-CSF improved survival, although full protection was not achieved (Fig 5). Mice surviving 80 hours after SEB challenge recovered fully from illness. Besides treatment 6 hours in advance, an additional preinjection of 5 μg of rhG-CSF was administered 18 hours before D-Gal/SEB injection, resulting in no better protective effect (Fig 5).

**T-cell marker analysis in vivo and proliferative responses in vitro.** To estimate whether rhG-CSF inhibits the binding of SAg to T cells, mice were treated with rhG-CSF (5 μg SC) 6 hours in advance and with SEB (10 μg IP), and dual color staining analysis was performed to follow the kinetics of Vβ8+ cells in lymph nodes and spleen. No significant change in clonal expansion or subsequent deletion of Vβ8+ cells among CD4+ or CD8+ T cells was detected in rhG-CSF–treated animals (data not shown).

The following experiments were performed to study whether rhG-CSF affects T-cell activation in vitro. Spleen cells, including some populations of mature and immature myeloid series, were stimulated in vitro with SEB, TSST-1, or Con A. Pretreatment with rhG-CSF failed to affect their proliferation in response to stimulation with either SAg or mitogen (data not shown). We then examined whether T-cell proliferation was affected in the presence of rhG-CSF. Spleen cells from naive mice were cultured with either SEB or TSST-1 in the presence of different concentrations of rhG-CSF (10−9 to 10−6 g/mL). T-cell proliferation in response to SAg was not influenced under these in vitro conditions (data not shown).

We additionally examined the various cytokine levels in conditioned media. Whole spleen cells were incubated with or without rhG-CSF for 20 hours in vitro and cytokine levels in the conditioned media were assayed. IL-2 levels in the conditioned media were not changed in the presence of rhG-CSF. Other cytokines, such as TNF-α, IFN-γ, IL-4, and IL-10, were also unaffected in vitro (data not shown). Treatment with G-CSF in vivo also did not lead to modulation of cytokine production in vitro in response to SAg or Con A (data not shown).

**DISCUSSION**

Hematopoiesis requires a highly complex series of cellular events in which a small population of stem cells needs to generate continuously large populations of maturing cells in various lineages. Under clinical and experimental conditions, reciprocal adjustments in lineage-specific cell production have been observed, suggesting that there are mechanisms that regulate a balance in multilineage hematopoiesis; the
upregulation of one cell lineage results in downregulation of another lineage. Viral infections induce T-cell activation and frequently cause a reduction in the ANC. In vivo administration of glucocorticoids results in lymphocytic suppression and noble granulocytosis. Chang et al. reported the reduction of BM lymphocytes in G-CSF-treated mice. TNF-α downregulates the expression of G-CSF receptor on neutrophils. In previous studies, other investigators have also suggested the involvement of granulopoietic agents in the suppression of primary lymphopoiesis both in vitro and in vivo. This inverse relationship in activating lymphoid cells and myeloid cells is substantiated by the present study. We have reported that mice pretreated with G-CSF were capable of developing resistance to T-cell-mediated lethal shock triggered by SAg. This protection was concomitant with diminished IL-2 levels in the bloodstream.

Although the mechanisms of septic shock are not fully understood, considerable evidence has been accumulated to indicate that increased permeability in combination with vasodilation are central events in its development. This enhanced permeability is largely the result of toxin-induced IL-2 production. In vitro analyses and animal models have demonstrated that the continued presence of IL-2 increases leukocyte-endothelial cell adhesion, which causes transendothelial hyperpermeability. In vivo, IL-2-activated cells may damage the endothelial cell layer and clinical administration of high doses of IL-2 has been frequently associated with systemic capillary leak syndrome. Indeed, a bolus injection of IL-2 causes septic-shock-like symptoms. Our study indicates that G-CSF protects mice against SAg shock, via suppression of systemic release of IL-2, and these results are consistent with those of previous studies that showed that treatment with rhG-CSF actually led to a decrease in LPS-induced pulmonary edema and alveolar capillary protein leakage in animals.

Several investigators have assumed that G-CSF-induced
neutrophilia might aggravate inflammatory lesions. However, all studies involving LPS- or live bacteria-induced diseases showed beneficial rather than deleterious effects of G-CSF. In common gram-positive bacterial infection, SAgs might be released in the local milieu and, concurrently, endogenous G-CSF would be released to promote the function and survival of neutrophils and contribute to a reduction in the toxic effects of SAg. In fact, serum levels of G-CSF are naturally increased during infection both in patients with and without neutropenia. However, when a large proportion of T cells are exposed to SAg at one time, as is the case of septic shock or TSS, endogenous G-CSF might be insufficient to oppose the lethal toxicity. Exogenous rhG-CSF may contribute to resist the toxicities of septic shock and TSS, even before microbiologic confirmation, via suppression of the macrophagic/lymphocytic system. In contrast, although GM-CSF can also accelerate myelopoietic recovery, GM-CSF has been shown to potentiate LPS toxicity. Thus, care should be taken in the clinical use of rhGM-CSF in severe infections.

As in the LPS-driven shock model, TNF seems to be the critical mediator because neutralizing anti-TNF MoAb protects mice from SAg-driven shock. Previous studies showed that the amount of serum TNF in SAg-challenged mice was up to five times greater than that in TSST-1-challenged mice, presumably because SAg-reactive Vβ8+ T cells represent a large fraction of all peripheral T cells compared with TSST-1-reactive Vβ15+ T cells. Pretreatment with rhG-CSF may not lead to achieve full protection against D-Gal/SEB challenge because of high levels of TNF. On the other hand, TNF is produced very early after SAg or LPS administration, but animals die several hours later, implying that this cytokine is unlikely to represent the final mediator in the pathophysiologic cascade. These observations indicate that TNF by itself is probably not sufficient to cause fatal shock and a synergistic mechanism with cytokines such as IL-1, IL-6, and IFN-γ as well as others is likely to induce the complicated pathway causing systemic shock. In septic shock, IL-1 acts directly on the blood vessels to induce vaso-dilation through the rapid production of platelet-activating factor and nitric oxide. Additionally, TNF and IL-1 act synergistically in inducing shock in mice and rabbits. Görgen et al. reported that G-CSF pretreatment protected rodents against LPS-induced toxicity via suppression of systemic TNF-α from macrophages. In our study, G-CSF pretreatment appeared to render mice resistant to SAg shock, with a reduction of systemic IL-2.

Anti-inflammatory agents are also released in response to SAgs. It is possible that G-CSF may induce anti-inflammatory substances leading to resistance against SAg-induced toxicity. IL-4 suppresses IL-1, TNF-α, and IL-6 secretion and upregulates the synthesis of IL-1 receptor antagonist. IL-10 inhibits cytokine production by T cells through its suppressive effect on macrophage APC function. Exogenous IL-10 was shown to be capable of protecting mice against SEB shock. Florquin et al. showed that pretreatment of mice with neutralizing anti-IL-10 MoAb before SEB challenge did not modify the release of TNF but led to increased serum IL-2 and IFN-γ levels. Another agent is endogenous glucocorticoid. In vivo, T-cell stimulation by bacterial SAg induces a rapid (peak at 90 to 120 minutes) increase in corticosterone serum levels, suggesting that endogenous glucocorticoids might control early T-cell activation. Although no upregulation of IL-10 or IL-4 was detected in this study, if rhG-CSF upregulates glucocorticoid secretion, it would suppress SAg-induced toxicity.

There are several possibilities concerning the mechanisms of G-CSF-mediated anti-inflammatory effects other than those we clarify here. Firstly, direct cell-to-cell interaction between activated neutrophils and antigen-presenting cells (APC)/T lymphocytes might transduce signals that suppress T-cell responses. The murine G-CSF receptor has been restricted to myelomonocytic cells and megakaryocytes. However, when cultured with myeloid cells of monocytic cells in vitro, rhG-CSF did not affect cytokine production from SAg-reactive T cells.

The second possibility is that G-CSF might alter the surface molecules that are essential for SAg binding or APC-T-cell interaction. The maximal T-cell response to its antigen requires presentation of the antigen by MHC class II molecule and the delivery of one or more costimulatory signals provided by heterotypic adhesion between receptor ligand pairs on the T cell and APC. We examined the changes of surface molecules such as B7.1, B7.2, intracellular adhesion molecule-1, and CD40 on B cells or macrophages as well as TCRβ or CD40 ligand on CD4+ or CD8+ T cells. Six hours after the administration of G-CSF, no definitive change in these molecules was observed after in vivo injection of rhG-CSF (data not shown).

Finally, G-CSF might directly or indirectly inhibit the receptor-ligand binding of TNF or other lethal effector molecules such as IFN-γ, IL-1, or IL-12, which are known to
potentiate the toxicity of TNF-α.\(^{19,21}\) Alternatively, G-CSF might induce some factors that specifically block the binding of these cytokines to their receptors, such as IL-1 receptor antagonist\(^{10}\) or soluble TNF receptor.\(^{12}\) Increased neutrophils bearing TNF receptors would also accelerate the consumption of TNF in serum, resulting in diminution of the deleterious effects of TNF on other tissues. Future experiments will aim to investigate these possibilities, and the use of G-CSF-deficient mice will contribute to the clarification of these mechanisms.\(^{45}\)

In conclusion, the present study provides important information concerning another beneficial effect of G-CSF. This is, to our knowledge, the first report showing G-CSF acts as a protective agent against T-cell-mediated lethal shock triggered by SAg. This protection was concomitant to diminished IL-2 production. The mechanisms of this have not been examined fully and further investigations must be performed. However, our findings may provide a clue to understanding the role of G-CSF in the host defense.

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