**Sulfur Mustard-induced Neutropenia: Treatment with Granulocyte Colony-stimulating Factor**

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**ABSTRACT**
See reprint.

**SUBJECT TERMS**
sulfur mustard, neutropenia, nonhuman primates, granulocyte colony-stimulating factor, G-CSF, treatment
Sulfur Mustard-Induced Neutropenia: Treatment with Granulocyte Colony-Stimulating Factor

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Although best known as a blistering agent, sulfur mustard (HD) can also induce neutropenia in exposed individuals, increasing their susceptibility to infection. Granulocyte colony-stimulating factor (G-CSF) and pegylated G-CSF (peg-G-CSF) have been approved by the U.S. Food and Drug Administration as hematopoietic growth factors to treat chemotherapy-induced neutropenia. The goal of this study was to determine the effectiveness of G-CSF and peg-G-CSF in ameliorating HD-induced neutropenia. African green monkeys (Chlorocebus aethiops) were challenged with HD and, at 1, 3, 5, or 7 days after exposure, G-CSF therapy (10 μg/kg per day for 21 days) was initiated. Peg-G-CSF (300 μg/kg, single treatment) was similarly tested, with treatment given at 3 days after exposure. Untreated HD-exposed animals recovered from neutropenia 28 days after exposure, whereas G-CSF- or peg-G-CSF-treated animals recovered 8 to 19 days after exposure (p < 0.05). These results indicate that G-CSF or peg-G-CSF may provide Food and Drug Administration-approved treatments that will reduce the duration of HD-induced neutropenia.

Introduction

Sulfur mustard (HD) is best known for its effects on epithelial tissues of the skin, eyes, or lungs. However, much of the HD dose passes into the circulatory system, resulting in systemic toxicity. Included in the systemic targets are bone marrow and the lymphatic system. Decreases in white blood cell counts were noted for casualties exposed to mustard during the Iran-Iraq war. The bone marrow injury consists of complete depletion of the granulocytic sites and degenerative changes in megakaryocytes, culminating in aplasia. Leukopenia, an abnormal reduction in circulating white blood cells (primarily lymphocytes and neutrophils), may result from bone marrow suppression or from recruitment of leucocytes from the bloodstream to sites of secondary infections (margination). HD-induced injury to the lymphatic system occurs before bone marrow damage, resulting in an initial reduction in circulating lymphocytes and then neutrophils.

Hematological data from rats exposed to HD by inhalation show consistent leukocyte suppression, primarily a loss of lymphocytes, at 24 hours after exposure. Gold and Scharf reported a significant increase in leukocyte counts for guinea pigs at 24 hours, followed by a decrease at 48 hours after subcutaneous exposure to HD. Many chemotherapeutic agents, including nitrogen mustard, also induce severe neutropenia, an excessive loss of circulating neutrophils that leaves patients susceptible to fever and infection.

Recombinant human granulocyte colony-stimulating factor (G-CSF) is a protein produced in Escherichia coli using recombinant DNA technology. It is a selective stimulator of pure granulocyte colonies from normal bone marrow. The most extensively studied clinical application of G-CSF has been in chemotherapy-induced myelosuppression, where it has been shown to reduce the duration of severe neutropenia, the duration of antibiotic therapy, and the length of hospitalization. The pegylated form of G-CSF (peg-G-CSF) has a longer half-life and more sustained duration of action than G-CSF. Both CSFs are Food and Drug Administration (FDA)-approved hematopoietic growth factors used clinically to treat chemotherapy-induced neutropenia. In a cooperative study with Amgen (Thousand Oaks, California), Navy researchers Meisenberg et al. tested a variety of compounds against both sulfur and nitrogen mustards, and showed that G-CSF significantly reduced the duration of nitrogen mustard-induced neutropenia in rhesus monkeys. However, this treatment compound has not been tested specifically against the chemical warfare compound HD. There is evidence in the literature of differences in the biological effects and therapeutic efficacies of treatment compounds, with respect to nitrogen mustard and HD. Pachura and Rall stated that, although hematopoietic depression is observed with HD, its degree and frequency are less than with nitrogen mustard. They attributed this to a more-direct effect of nitrogen mustard on bone marrow when given systemically. In comparing HD and two nitrogen mustards, Kindred noted that all three compounds tested induced neutropenia and lymphocytopenia. However, the timing and extent of these changes differed among the three mustards. Rappeneau et al. tested a variety of compounds against both sulfur and nitrogen mustard toxicity in bronchial epithelial cells. They found that, even for effective compounds, the level of protection against HD was always weak, compared with that against nitrogen mustard. Gray et al. saw that tetracycline therapy was significantly more effective in treating nitrogen mustard-exposed thymocytes than HD-exposed thymocytes. For these
reasons, we thought it would be worthwhile to investigate the efficacy of G-CSF for treatment of HD-induced myelosuppression. Presented here are data on the hematological profile of HD exposure, with or without G-CSF or peg-G-CSF treatment, for up to 30 days after exposure, to determine the efficacy of these compounds in ameliorating HD-induced neutropenia. The advantage to the Army of testing FDA-approved drugs is much faster fielding and deployment of the products should they prove effective. If neutrophil counts can be returned to control levels sooner, then secondary infections may be reduced. This could ultimately decrease the duration of antibiotic therapy for soldiers with secondary infections and the length of patient hospitalization.

**Methods**

**Animals**

Fifty-six male African green monkeys (AGMs) (*Chlorocebus aethiops*), ranging in weight from 4 kg to 7.75 kg, were used in this study. In conducting the research described in this report, we adhered to the Guide for the Care and Use of Laboratory Animals by the Institute of Laboratory Animal Resources, National Research Council, in accordance with the stipulations mandated for an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility.

**Blood Sampling and Processing**

AGMs were anesthetized with ketamine (10 mg/kg, administered intramuscularly) and weighed; blood samples (2 mL) were drawn from the saphenous vein into ethylenediaminetetraacetic acid-treated tubes and mixed. Samples were then analyzed for complete blood count and cell differential count with a Cell Dyne 3500 hematology analyzer (Abbott Diagnostics, Santa Clara, California).

**HD Exposure**

Animals were anesthetized with ketamine (15 mg/kg, administered intramuscularly) and weighed; a 22-gauge catheter was introduced into the saphenous vein for HD administration. HD (purity, >97%) was obtained from the Edgewood Chemical Biological Center (Aberdeen Proving Ground, Maryland). The HD working stock solution was made up in absolute ethanol, at a concentration of 9.5 mg/mL. Immediately before the intravenous injection, the HD was diluted in saline solution to the appropriate concentration and injected at 1.0 mL/kg. The HD was administered as two half-dose injections, each infused over 2.5 minutes with a microprocessor-controlled infusion pump (model 200P; Stoelting, Wood Dale, Illinois). The half-time for hydrolysis of HD in saline solution at room temperature is 30.3 minutes; therefore, each injection was made from a fresh dilution. The average time from start of dilution to completion of HD injection was 6 minutes. Each mustard injection was followed by a 1.0-mL saline flush.

**HD Ranging Study**

The initial HD exposures were to establish a HD dose that reproducibly induced significant neutropenia (absolute neutrophil counts [ANCs] of <1,000 cells per μL for ≥5 days). Nine monkeys (n = 3 per dose) were treated intravenously with one of three doses of HD (0.75, 1.0, or 1.5 mg/kg), to determine the effects of HD on the hematological profile over time. Blood samples were collected from these animals immediately before HD exposure and 3 to 4 days per week for up to 5 weeks after HD exposure.

**G-CSF Evaluation**

The goal of this work was to determine whether G-CSF alleviates HD-induced neutropenia and how long treatment can be delayed and still be effective. Recombinant human G-CSF (Neupogen) was graciously supplied by Amgen, under a Material Transfer Agreement. The dose and the dosing frequency selected were recommended by Amgen and were in the range of those recommended for use as a chemotherapy adjunct. G-CSF therapy was initiated 1, 3, 5, or 7 days after exposure (n = 4-7 per group). Parallel HD-only and G-CSF-only control groups (n = 6) were also included. A single dose of HD (1.0 mg/kg, administered intravenously) was used for exposure. Table I shows the timing of HD administration in relation to the beginning of G-CSF treatment. Blood samples were collected from animals receiving both HD and G-CSF immediately before and 1, 3, 5, and 7 days after exposure (depending on the G-CSF group) and periodically thereafter for 30 days after G-CSF treatment initiation. Blood samples were collected from HD control and G-CSF control

| TABLE I  
<p>| HD EXPOSURE, G-CSF DOSING, AND BLOOD DRAW SCHEDULE FOR EACH OF THE SIX TREATMENT GROUPS | Day after G-CSF (or HD for HD Control Group) |</p>
<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Days before G-CSF</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD only</td>
<td>-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>G-CSF only</td>
<td>-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>G-CSF day 1</td>
<td>-7</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>G-CSF day 3</td>
<td>-7</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>G-CSF day 5</td>
<td>-7</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td>x</td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>G-CSF day 7</td>
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<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
<td>x</td>
</tr>
</tbody>
</table>

HD indicates day of dosing with sulfur mustard; G-CSF, daily G-CSF injections initiated; x, blood sample drawn. All G-CSF dosing began on day 0. The arrows on the right indicate that daily G-CSF dosing and blood sampling continued, as described in the text. The sample size for each group is given in parentheses.
animals immediately before HD or G-CSF administration and periodically thereafter for 30 days.

Pegylated G-CSF Evaluation

Pegylated recombinant methionyl human G-CSF (Neulasta) was also provided by Amgen, under a Material Transfer Agreement, in 1-mL vials at 10.0 mg/mL. It was administered subcutaneously once at a dose of 300 μg/kg, as recommended by Amgen. Peg-G-CSF therapy was given 3 days after exposure (n = 6). Three AGMs receiving HD only and three receiving HD plus G-CSF beginning 3 days after exposure were studied in parallel with the peg-G-CSF-treated animals. A single dose of HD (1.0 mg/kg, administered intravenously) was used for exposure. Blood samples were collected from animals receiving HD plus peg-G-CSF or HD plus G-CSF immediately before and 1 and 3 days after exposure and periodically thereafter for 30 days after treatment initiation. Blood samples were collected from HD-only animals immediately before HD exposure and periodically thereafter for 30 days.

Data Analysis

One-factor analysis of variance was used, followed by Tukey’s multiple-comparison test if significant treatment group or study day differences were observed. All statistical tests were conducted at the α = 0.05 level.

Results

HD Ranging Study

Figure 1 shows the average hematological profiles (total white blood cells, neutrophils, and lymphocytes; n = 3 per dose) for the animals receiving 0.75, 1.0, and 1.5 mg/kg HD. The lymphocyte counts reached a nadir of 570 cells per μL (79% decrease) for 0.75 mg/kg, 460 cells per μL (78% decrease) for 1.0 mg/kg, and 133 cells per μL (93% decrease) for the 1.5 mg/kg HD dose ~4 days after exposure. A reduction in lymphocytes (~70%) was typically observed 1 day after exposure. The ANC nadir was typically reached 7 to 9 days after exposure and was 354 cells per μL (86% decrease) for the 0.75 mg/kg dose, 68 cells per μL (97% decrease) for the 1.0 mg/kg dose, and 8 cells per μL (99.7% decrease) for the 1.5 mg/kg dose. As discussed below, the 1.0 mg/kg intravenous dose of HD was selected for the remaining studies, based on the degree and duration of neutropenia.

G-CSF Evaluation

Animals were challenged with 1.0 mg/kg HD, administered intravenously, and G-CSF treatment was initiated 1, 3, 5, or 7 days after exposure. In Figure 2, the ANC values for the four treatment groups, relative to the two control groups (HD only and G-CSF only), are shown. Table II shows that the ANC nadir was typically reached 5 to 9 days after exposure. The ANC was observed on day 5. It can be seen in Table II that, the longer treatment was delayed, the lower was the nadir. All G-CSF treatment groups recovered from HD-induced neutropenia faster than did the untreated control animals (HD only). Untreated animals did not recover from HD-induced neutropenia until day 28 after exposure, whereas the G-CSF-treated animals recovered 8 to 19 days after exposure (Fig. 2). The duration of neutropenia and severe neutropenia (i.e., the number of days with ANC of < 1,000 cells per μL or < 500 cells per μL, respectively) for each treatment group is shown in Table II. G-CSF treatment significantly (p < 0.05) shortened the duration of neutropenia, relative to untreated animals.

Pegylated G-CSF Evaluation

In this experiment, peg-G-CSF was administered subcutaneously once at a dose of 300 μg/kg, on day 3 after exposure (n = 6).
After reaching a nadir 5 to 9 days after exposure, the ANC for peg-G-CSF-treated animals responded very rapidly to untreated HD-exposed animals vs. 6.3 days for rhesus monkeys. The other treatment delays were also evaluated. The ANC for peg-G-CSF-treated animals did not recover from neutropenia, whereas the untreated control animals recovered significantly faster from neutropenia. All of the four treatment groups recovered from neutropenia faster than the HD-only animals. However, it is not possible to determine whether this is a species difference, a result of the different mustards used, or a combination of these two factors.

In the main portion of the study, we wanted to determine whether G-CSF would alleviate HD-induced neutropenia, as well as how long the treatment could be delayed and still maintain efficacy. It can be seen in Figure 2 that G-CSF effectively alleviated HD-induced neutropenia. All of the four treatment groups recovered from neutropenia faster than did the HD-only control group and returned to baseline ANC levels faster than did the untreated control animals. However, it is not possible to statistically discriminate among any of the four treatment delays (1, 3, 5, or 7 days). The 7-day animals had effectively reached their ANC nadir when treatment began, and these animals immediately began to recover from neutropenia after treatment initiation. The ANC nadir for each of the other groups was reached after G-CSF treatment was initiated, but recovery from neutropenia for each group was faster than that for untreated animals. These results were comparable to the observations of Meisenberg et al.13

Interestingly, concomitant with a marked decrease (~70%) in lymphocytes observed 1 day after exposure, there was usually a transient increase in ANC. Despite this initial influx of neutrophils, the ANC began to decrease by day 4, reaching the nadir 5 to 9 days after exposure. A second spike in ANC was generally observed -1 day after the start of G-CSF administration. This rapid post-G-CSF spike is thought to be attributable to the release of reserve neutrophils/precursors from the bone marrow (left shift). After reaching a nadir 5 to 9 days after exposure, the G-CSF-treated animals recovered significantly faster from neutropenia than did their untreated cohorts. The group with the shortest duration of neutropenia, the day 1 group (ANC of < 1,000 for 6.2 days) (Table II), was treated at the time recommended by the American Society of Clinical Oncology for G-CSF used as a chemotherapy adjunct.19 In a military setting, it may not be possible to initiate treatment 1 day after exposure; therefore, the other treatment delays were also evaluated.

For the evaluation of peg-G-CSF, the single treatment inject-
### TABLE II

**MEAN ANC NADIR, DAYS AFTER HD TO NADIR, AND DURATION OF HD-INDUCED NEUTROPENIA ACCORDING TO TREATMENT GROUP**

<table>
<thead>
<tr>
<th>G-CSF Treatment Group</th>
<th>Sample Size</th>
<th>Mean ANC Nadir (Cells per µL)</th>
<th>Days after HD to Nadir</th>
<th>Mean No. of Days with ANC of &lt;1000 cells per µL</th>
<th>Mean No. of Days with ANC of &lt;500 cells per µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 7</td>
<td>6</td>
<td>254</td>
<td>7</td>
<td>8.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 5</td>
<td>4</td>
<td>275</td>
<td>9</td>
<td>14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 3</td>
<td>7</td>
<td>359&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
<td>10.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Day 1</td>
<td>5</td>
<td>372&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5</td>
<td>6.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Peg (3 day)</td>
<td>5</td>
<td>702&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7</td>
<td>13.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HD only</td>
<td>11</td>
<td>143</td>
<td>7</td>
<td>23.6</td>
<td>18.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Significantly different from HD only.

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**Fig. 3.** ANCts in AGMs exposed to HD (1.0 mg/kg, administered intravenously) on day 0 and treated with G-CSF (10 µg/kg, administered subcutaneously daily for 21 days) or peg-G-CSF (single 300 µg/kg subcutaneous injection) beginning on day 3 after exposure. The vertical dotted line denotes the initial G-CSF treatment. The horizontal dotted lines denote significant (<1,000 cells per µL) or severe (<500 cells per µL) neutropenia.

**Fig. 4.** Effect of HD on average lymphocyte counts in AGMs exposed to HD (1.0 mg/kg, administered intravenously) on day 0 and treated with G-CSF (10 µg/kg, administered subcutaneously daily for 21 days). The G-CSF-only group was not exposed to HD.

**Fig. 5.** Effect of HD on average RBC counts in AGMs exposed to HD (1.0 mg/kg, administered intravenously) on day 0 and treated with G-CSF (10 µg/kg, administered subcutaneously daily for 21 days). The G-CSF-only group was not exposed to HD.

**Fig. 6.** Effect of HD on average platelet counts in AGMs exposed to HD (1.0 mg/kg, administered intravenously) on day 0 and treated with G-CSF (10 µg/kg, administered subcutaneously daily for 21 days). The G-CSF-only group was not exposed to HD.

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The biological effect of peg-G-CSF is prolonged in a neutropenic condition, and the drug is slowly eliminated as neutrophil numbers increase. This self-regulating design of the American Society of Clinical Oncologists as severe neutropenia (<500 cells per µL). The peg-G-CSF ameliorated the HD-induced neutropenia as fast as or faster than G-CSF, but the effect was not fully maintained. The mean ANC for the peg-G-CSF-treated group fluctuated around 1,000 cells per µL from day 10 through day 21 after HD (Fig. 3). With pegylation of the molecule, renal clearance is minimized and neutrophil-mediated endocytosis is the predominant route of elimination, which gives peg-G-CSF a much longer circulating half-life than G-CSF. Therefore, the biological effect of peg-G-CSF is prolonged in a neutropenic condition, and the drug is slowly eliminated as neutrophil numbers increase. This self-regulating design of

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peg-G-CSF may be providing a sufficient number of neutrophils to combat infection in this species. In clinical applications, peg-G-CSF is given once per chemotherapy cycle and provides results comparable to ~11 daily doses of G-CSF. By 10 days after exposure, these peg-G-CSF-treated AGMs were well positioned for follow-up therapy. In this study, only one dose of therapy was given. In addition to the less severe neutropenia, another noteworthy advantage of peg-G-CSF is the sustained effect with a single treatment.

In a clinical setting, the use of hematopoietic growth factors such as G-CSF and peg-G-CSF allows clinicians to reduce the impact of neutropenia without the need to modify chemotherapy doses as a result of myelotoxicity. Higher doses of chemotherapy agents can be used, increasing the odds of success, when these growth factors are used as an adjunct. In his exhaustive work describing casualties from the Iran-Iraq conflict, Willems noted that HD casualties with ANC of <200 had extremely high mortality rates; therefore, recognition and prompt treatment of neutropenia are paramount.

HD exposure also had profound effects on cell types other than neutrophils, including lymphocytes, platelets, and RBCs. G-CSF treatment did not have any effect on these cells or their recovery. The chronology of hematologic effects shows that lymphocytes are the first cell type to show a decrease in numbers, followed by neutrophils, platelets, and RBCs. Sidell and Hurst reported a similar chronology following mustard exposure in a review of clinical reports of battlefield exposures, as did Willems. As Meisenberg et al. reported and as corroborated here, the drop in lymphocytes is predictive of impending neutropenia. Platelet counts should also be carefully monitored, because rather pronounced thrombocytopenia was generally seen. Although there have been reports of G-CSF exacerabting thrombocytopenia, that did not appear to be the case in this study, because there were no significant differences (p > 0.05) in platelet counts for any of the HD-exposed groups. Harker et al. described a combination of pegylated recombinant human megakaryocyte growth and development factor in combination with G-CSF as a treatment for hepsulfam-induced thrombocytopenia and neutropenia.

In conclusion, these results indicate that both G-CSF and peg-G-CSF may provide a FDA-approved treatment that can reduce the duration of HD-induced neutropenia. Treatment with either of these compounds could ultimately decrease the duration of antibiotic therapy for casualties with secondary infections, leading to increased survival rates and reduced length of patient hospitalization. The facts that G-CSF and peg-G-CSF are FDA approved for a closely related clinical application and are commercially available could ultimately reduce the time for fielding a product that could be beneficial in the medical management of HD casualties.

Acknowledgments
We recognize the outstanding support of the veterinary technicians of the Comparative Medicine Division who assisted with this work.

References