Modulation of efficacies and pharmacokinetics of antibiotics by granulocyte colony-stimulating factor in neutropenic mice with multidrug-resistant Enterococcus faecalis infection

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It has been demonstrated previously that, in non-neutropenic animals, interferon-gamma markedly enhances the efficacies of gentamicin and vancomycin against Enterococcus faecalis resistant to these antibiotics. The aim of our study was to determining whether granulocyte colony-stimulating factor (G-CSF) can be beneficial as an adjunct to gentamicin and vancomycin in the treatment of the same infection in neutropenic mice. After induction of neutropenia by cyclophosphamide, mice were inoculated ip with the organism. The infected animals received sc administrations of G-CSF, antibiotic or a combination of both agents at determined dosing regimens. Infected animals treated with G-CSF alone showed a dose-dependent increase in survival. The inoculum size used in establishing infection affected the effectiveness of the cytokine. Survival was significantly better in the infected animals given gentamicin and vancomycin plus G-CSF than in those given antibiotics or G-CSF alone. The possibility of pharmacokinetic interaction between G-CSF and each of the antibiotics was examined. The cytokine significantly increased the plasma clearance of gentamicin, with a resultant decrease in the area under the concentration–time curve (AUC), while the disposition of vancomycin was not affected. This study suggests that G-CSF may be a useful adjunct to gentamicin and vancomycin for the treatment of multidrug-resistant E. faecalis infection in neutropenic patients.

Introduction

The treatment of serious enterococcal infections has been complicated by increasing resistance of enterococci and the emergence of multidrug-resistant strains (MDRE). There are limited therapeutic options available for treatment of infections with these strains. The need to find other ways of treating MDRE infections prompted our earlier study which showed that interferon-gamma (IFN-γ) markedly increased the efficacy of gentamicin or vancomycin in non-neutropenic mice infected with gentamicin- and vancomycin-resistant Enterococcus faecalis (GVRE). However, in mice with induced neutropenia, there was no beneficial effect of IFN-γ in combination with these antibiotics, suggesting that the activity of IFN-γ is principally mediated by neutrophils. Thus, it is reasoned that to combat GVRE infections in neutropenic hosts by immunotherapy, the immunomodulator should be able to stimulate the production of neutrophils and enhance the neutrophil’s functional activity.

Granulocyte colony-stimulating factor (G-CSF) is a haematopoietic growth factor that promotes the proliferation and differentiation of progenitors of polymorphonuclear neutrophils. One of its effects on neutrophils is to potentiate their phagocytic and microbicidal activities against a variety of organisms, including fungi and Gram-positive and -negative microbes. The activity of G-CSF-stimulated leucocytes against enterococci has not been reported. Adjunctive G-CSF enhances antibiotic therapy in experimental infections in neutropenic and non-neutropenic animals. The results obtained using these models have encouraged trials of G-CSF–antibiotic combination therapy in refractory infections in neutropenic humans, and improved resolution of infections has been reported. Thus, augmentation of the immune system with G-CSF seems to be a promising new approach to
adjunctive therapy of refractory infections. Hence, it was thought worthwhile investigating whether G-CSF enhances the effectiveness of gentamicin and vancomycin in neutropenic mice with GVRE infection. G-CSF has been shown to alter the disposition of other drugs by decreasing their plasma clearance. Since gentamicin and vancomycin have narrow therapeutic windows, it was also of interest to examine the effect of the cytokine on the pharmacokinetics of these antibiotics.

Materials and methods

Animals

Female Swiss Webster mice weighing 20–25 g (Harlan Sprague Dawley, Indianapolis, IN, USA) were used. They were acclimatized for 1 week before the study, in a room with controlled temperature and a 12 h light–12 h dark cycle. The animals were allowed access to food and water ad libitum.

Bacteria

A clinical isolate of *E. faecalis* resistant to vancomycin (MIC 16 mg/L) and gentamicin (MIC > 256 mg/L) was used. The organism was subcultured on to trypticase soy blood agar (TSBA; BBL Microbiology Systems, Cockeysville, MD, USA) at 37°C for 24 h before use. Bacteria taken from the TSBA plate were cultured overnight in Mueller–Hinton broth (BBL), diluted in heat-sterilized mucin suspension to a final mucin concentration of 3% (w/v) and used for inoculation of the mice.

Drugs

Gentamicin sulphate and vancomycin hydrochloride (Sigma Chemical Co., St Louis, MO, USA) were dissolved in normal saline at the time of use. The antibiotics were administered sc to mice in a volume of 0.2 mL.

Cyclophosphamide (Mead Johnson Pharmaceuticals, Evansville, IN, USA) was reconstituted according to the manufacturer’s instructions, diluted in normal saline to obtain the required dosages and administered ip in 0.2 mL volumes.

Recombinant human G-CSF (rhG-CSF) (Neupogen; Amgen Biologicals, Thousand Oaks, CA, USA) was stored at 4°C until use; it was diluted as required in normal saline. It was administered sc to mice in 0.2 mL volumes.

Induction of infection

Mice were rendered neutropenic by ip administration of cyclophosphamide 150 and 100 mg/kg 4 days and 1 day, respectively, before infection or any further treatment (day 0). Infection was established by ip inoculation of neutropenic mice with 0.5 mL of bacterial suspension prepared as described above. To determine the MLD, groups of 10 mice were infected with serial dilutions of the bacterial suspension; the lowest dilution of the inoculum that caused 100% mortality within 3 days after infection was recorded. The infectious dose that produced about 80% mortality (LD80) was also determined.

Therapy with G-CSF

To evaluate the anti-enterococcal activity of G-CSF, neutropenic animals were inoculated with 0.5 mL of c. 4 × 10^7 cfu/mL (the MLD) of the organism. They were randomized into treatment groups (20 or 24 mice/group) which received 0–300 µg/kg/day sc doses of G-CSF for 3 days, starting immediately after infection. The experiment was repeated with animals receiving a lower inoculum (0.5 mL of 2 × 10^6 cfu/mL) that resulted in 80% mortality. Percentage survival, as a measure of efficacy, was recorded daily for 5 days after infection. The experiment was repeated and pooled data were used for statistical analysis.

Combination therapy with antibiotics and G-CSF

Before evaluating the effect of G-CSF combined with antibiotics, the doses of vancomycin and gentamicin associated with a maximal efficacy but not more than 50% survival of the infected animals were determined as follows: groups of neutropenic mice were infected with an LD80 of the organism; 1 h after infection, vancomycin was administered sc at a dose range of 0.8–12.8 mg/kg/day (doses of 0.8, 1.6, 3.2, 6.4 and 12.8 mg/kg/day) or gentamicin at a dose range of 2–64 mg/kg/day (doses of 2, 4, 8, 16, 32 and 64 mg/kg/day). A single dose of vancomycin was given whereas two doses of gentamicin were administered. Sixteen mice were used for each drug dose and the animals were observed for survival for 5 days after infection.

To evaluate combination therapy, mice infected with an LD80 of the organism were randomized into treatment groups which were given sc gentamicin alone (16 mg/kg/day for 2 days), vancomycin alone (2 mg/kg/day for 1 day), G-CSF alone (300 µg/kg/day for 3 days), gentamicin plus vancomycin or G-CSF plus either or both antibiotics. All doses were given in 0.2 mL volumes. Infected animals that received only normal saline served as a control group. G-CSF was administered immediately after infection and the antibiotics 1 h later. In combination therapy, the agents were given at the same dosages as in monotherapy. Thirty to 50 mice were used in each treatment group and the animals were monitored daily for survival over 5 days.

Pharmacokinetic studies

To assess whether G-CSF influences the disposition of vancomycin or gentamicin, the pharmacokinetics of each antibiotic were determined in the presence and absence of G-CSF. Two groups of neutropenic mice (32 mice/group)
received three sc doses of G-CSF (300 μg/kg/day), starting on day 0 following induction of neutropenia. Twenty-four hours after the last G-CSF dose, the animals were inoculated with an LD$_{50}$ of the organism. One hour after inoculation, the animals were injected sc with single doses of vancomycin 40 mg/kg or gentamicin 40 mg/kg. Another two groups of mice (32 mice/group) received similar treatments except that G-CSF administration was replaced with normal saline. Blood was obtained from the mice by intracardiac puncture 0.17, 0.33, 0.75, 1.0, 1.5, 2.0, 3.0 and 4.0 h after antibiotic administration. Four mice were used for each time point. The blood was centrifuged at 2000g for 10 min to obtain serum, which was transferred into separate polypropylene tubes and stored at –80°C until analysis.

The gentamicin and vancomycin concentrations in the serum samples were assayed by a fluorescence polarization immunoassay procedure (Cobas Integra; Roche Diagnostic Systems, Somerville, NJ, USA). The lower limits of detection of the assay were 0.14 mg/L for gentamicin and 1.3 mg/L for vancomycin. The within- and between-run coefficients of variation were <2% at concentrations of 5 and 30 mg/L for gentamicin and vancomycin, respectively.

The means of four individual concentrations at each time point were used for pharmacokinetic evaluation. The pharmacokinetic parameters—apparent volume of distribution ($V_d$), area under the serum drug concentration–time curve (AUC), maximum serum drug concentration ($C_{max}$), terminal elimination half-life ($t_{1/2}$), and total body clearance ($Cl_T$) were computed employing non-linear least-square techniques (PCNONLIN version 4.2; Statistical Consultants, Lexington, KY, USA). Compartment model selection was based on visual inspection of the fit and use of the correlation between the observed and the PCNONLIN-calculated concentrations. Other criteria, including Akaike’s information criterion (AIC), were used to discriminate between models; a model having the largest AIC was considered better. A two-compartment open model with iv bolus input and first-order elimination rate from the central compartment was used to characterize gentamicin pharmacokinetics. The concentration–time profiles of the vancomycin in the presence and absence of G-CSF overlapped, so it was not thought necessary to generate pharmacokinetic parameters for it.

### Statistical analysis

The minimum number of animals required in each treatment group to provide sufficient statistical power was determined by sample size estimation based on the log rank test for a fixed time and constant hazard ratio. The percentage survival obtained following treatments of the infected neutropenic mice with different regimens was compared using Fisher’s exact method. Difference in pharmacokinetic values of gentamicin following administration of the drug alone and with G-CSF was analysed by Student’s t-test. For all tests a $P$ value < 0.05 was considered significant.

### Results

#### Treatment effect of G-CSF

The ability of G-CSF to affect the survival of neutropenic mice infected with GVRE is presented in Table I. Treatment

<table>
<thead>
<tr>
<th>Infectious dose</th>
<th>G-CSF dose* (μg/kg/day)</th>
<th>Number of mice (%) surviving after infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>day 1</td>
</tr>
<tr>
<td>LD$_{100}$</td>
<td>0</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>24</td>
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<tr>
<td></td>
<td>200</td>
<td>24</td>
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<tr>
<td></td>
<td>300</td>
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<tr>
<td>LD$_{50}$</td>
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<td>20</td>
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<tr>
<td></td>
<td>10</td>
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<tr>
<td></td>
<td>200</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>300^b</td>
<td>24</td>
</tr>
</tbody>
</table>

*Animals received three daily sc doses of G-CSF in 0.2 mL normal saline starting immediately after infection.

^bFrom day 3 after infection, survival in this group was significantly higher than that in the group receiving no G-CSF ($P < 0.05$).
Efficacy of G-CSF–antibiotic combination therapy

The therapeutic effect of combined administration of G-CSF and gentamicin in the treatment of GVRE-infected neutropenic mice is depicted in Figure 1. The doses of gentamicin and G-CSF that maximally improved survival were chosen based on the results of dose range studies with each agent. From day 4 after infection, treatment with gentamicin alone (16 mg/kg/day for 2 days), G-CSF alone (300 μg/kg/day for 3 days) or a combination of both agents at the same dosages as used separately, resulted in survival of 30%, 53% and 73%, respectively. Compared with the control group, treatment with gentamicin alone produced a non-significant increase in survival \(P > 0.05\), but the efficacy was markedly enhanced by the addition of G-CSF to the antibiotic therapy \(P < 0.001\). Survival of mice treated with G-CSF and gentamicin was also greater \(P = 0.05\) than that of mice treated with the cytokine alone.

The results of treatment of the GVRE-infected mice with vancomycin alone or in combination with G-CSF, with or without gentamicin, are depicted in Figure 2. At the end of the 5 day survival monitoring period, treatment with G-CSF (300 μg/kg/day for 3 days), vancomycin (2 mg/kg/day for 1 day) or a combination of both agents at the same dosages as in monotherapy resulted in 48%, 50% and 65% survival, respectively. This indicated that G-CSF increased the efficacy of vancomycin, though not significantly \(P > 0.05\), in the treatment of GVRE infection in neutropenic animals. Addition of gentamicin (16 mg/kg/day for 2 days) to the G-CSF–vancomycin therapy resulted in 80% survival on day 3 after infection; this was markedly higher \(P < 0.01\) than survival in the groups that received G-CSF and vancomycin (52% survival) or G-CSF alone.
G-CSF–antibiotic combination against *E. faecalis*

**Pharmacokinetic interaction studies with G-CSF**

Figure 3 shows the influence of co-administration of G-CSF on the pharmacokinetics of gentamicin or vancomycin in GVRE-infected neutropenic mice. There was an overlap of the kinetic profiles of vancomycin following administration of a single dose of the drug, with or without G-CSF, indicating that G-CSF did not alter the disposition of vancomycin. In contrast, the kinetics of gentamicin were significantly different when it was administered with G-CSF. This is evident in Table II, which shows that the cytokine caused a significant increase in $\text{Cl}_T$ with a resultant decrease in AUC of gentamicin ($P < 0.05$).

**Discussion**

The incidence of enterococcal infections has increased greatly in recent years and the emergence of multidrug-resistant strains of the organism is of great concern.25,26 The present study furthers our earlier investigations which demonstrated that immunochemotherapy is a promising approach for combating MDRE infections.5,27 IFN-γ is not effective against enterococcal infection in neutropenic patients, and there is high mortality due to MDRE infections in such patients,5,28,29 so an effective way of treating these patients is needed.

Treatment with G-CSF showed a dose-dependent beneficial effect on the survival of infected animals (Table I). Survival was monitored over 5 days because infected animals surviving for 3 days were generally long-term survivors. Similar dose-dependent antimicrobial-induced activity of G-CSF has been observed in other neutropenic animal infection studies.13,30,31 Although a significant improvement in survival has been produced with a G-CSF dose as low as 10 μg/kg in the treatment of other bacterial infections,30 similar low doses had no effect in this study. This is probably attributable to the degree of neutropenia and/or level of infectious burden, as these factors affect the efficacy of G-CSF. Findings in this study (Table I) are consistent with results of other investigations, which demonstrate that the infectious dose of an organism is an important determinant of G-CSF treatment outcome.32 Another plausible explanation of why G-CSF’s effects in this study were manifested at relatively high doses may be related to the known variation in susceptibility of different organisms to the G-CSF-augmented antibacterial activity of neutrophils.9 In some studies, a G-CSF dose as high as 100 mg/kg was effective in experimental infections.18

This study demonstrates the value of G-CSF when administered in combination with gentamicin and vancomycin in the treatment of GVRE infection. That G-CSF enhances the effectiveness of antimicrobial therapy of a wide variety of infections has been demonstrated in neutropenic animal models and validated in refractory infections in humans.16,17,20,22,33 Enhancement of granulopoiesis, mobilization of mature neutrophils from the marrow storage pool into circulation and enhancement of the activity

**Table II.** Pharmacokinetics (± S.E.M.) of gentamicin in neutropenic *E. faecalis*-infected mice after a single sc administration of 40 mg/kg dose of the drug, with or without G-CSF

<table>
<thead>
<tr>
<th>Drug</th>
<th>$C_{\text{max}}$ (mg/L)</th>
<th>AUC (mg·h/L)</th>
<th>$t_{1/2\beta}$ (h)</th>
<th>$V_d$ (L/kg)</th>
<th>$\text{Cl}_T$ (L/h/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gentamicin</td>
<td>74.0</td>
<td>86.8 (4.8)</td>
<td>0.60 (0.03)</td>
<td>0.37 (0.04)</td>
<td>0.46 (0.04)</td>
</tr>
<tr>
<td>Gentamicin + G-CSFa</td>
<td>74.2</td>
<td>66.8 (4.2)</td>
<td>0.52 (0.03)</td>
<td>0.45 (0.04)</td>
<td>0.60 (0.03)</td>
</tr>
</tbody>
</table>

*aMice received daily doses of 300 μg/kg of G-CSF for 3 days; 24 h after the third and last dose of G-CSF, gentamicin was administered 1 h after inoculation of bacteria.
of mature neutrophils have been shown to be responsible for the protective effects exerted by G-CSF against otherwise lethal infections in granulocytopenic animals.\textsuperscript{16,30} The rapidity of these G-CSF effects may explain the observed efficacy of the cytokine when administered therapeutically, as in this study.\textsuperscript{34,35}

Although the addition of gentamicin did not modify the effect of treatment with vancomycin, the greatest improvement in survival of the GVRE-infected mice was achieved by giving both antibiotics in combination with G-CSF (Figure 2). The efficacy of the triple combination therapy may be attributable to a possible additive effect of both antibiotics on bacterial alteration, as this could further enhance the organism’s susceptibility to the action of the G-CSF-activated leucocyte. Exposure of Gram-positive bacteria to subinhibitory levels of antibiotics, including vancomycin and gentamicin, is known to increase the sensitivity of the organisms to the microbicidal activity of neutrophils.\textsuperscript{36} Combination therapy with G-CSF and both antibiotics is of clinical relevance since treatment with a cell wall active antibiotic and an aminoglycoside is already established as a standard therapy for enterococcal infections.\textsuperscript{37} The doses of the agents used in this study were selected so as to produce an intermediate level of survival of the infected animals. This design allowed the determination of a beneficial effect of G-CSF on survival beyond that achievable with antibiotic treatment alone. The G-CSF once-daily dosing regimen was intended to synchronize with the effects of a single dose of the cytokine on neutrophil number and function which lasts approximately 24 h, as observed in animals and humans.\textsuperscript{30,38} It is pertinent to note that the G-CSF dose used in this study, 300 $\mu$g/kg/day, is much higher than the usual clinical dose of the cytokine (5 $\mu$g/kg/day). However, with the lower infectious load expected in humans, and given that the efficacy is inoculum dependent, a high dose of the cytokine may be clinically unnecessary.

Various cytokines, including G-CSF, have been shown to alter the kinetics of other concomitantly administered drugs.\textsuperscript{23,39} Hence, we examined whether G-CSF modifies the disposition of gentamicin and vancomycin, which could result in toxic concentrations of the antibiotics. The observation that G-CSF increased the plasma clearance of gentamicin with a resultant decrease in AUC (Table II) is not consistent with the reported effect of the cytokine in decreasing drug clearance.\textsuperscript{23} This led the authors to suggest that, like other inflammatory cytokines, G-CSF can impair hepatic oxidative drug metabolism.\textsuperscript{23} Since gentamicin elimination is primarily by a non-metabolic route, a process involving inhibition of metabolism cannot explain the increased clearance observed in the present study. In any case, inhibition of metabolism is an unlikely explanation for increased clearance even for a drug that is cleared hepatically. Gentamicin was also eliminated more quickly when administered with IFN-$\gamma$.\textsuperscript{5} It was hypothesized that the change in disposition of gentamicin was probably caused by multiple organ effects and/or capillary leak syndrome caused by IFN-$\gamma$. At standard clinical doses, G-CSF is not known to cause significant capillary leak syndrome, but its haemodynamic effects at doses as high as 300 $\mu$g/kg/day have not been reported. For drugs that accumulate within neutrophils, increased leucocytosis, induced by G-CSF, can alter the disposition of such drugs, since neutrophils can serve as secondary drug transport systems.\textsuperscript{38,40} This may not apply in this case because phagocytes take up gentamicin poorly and neutropenia has not been shown to modify the disposition of gentamicin in humans.\textsuperscript{41} However, the possibility that G-CSF-induced neutrophil recovery can influence the disposition of gentamicin in our model cannot be ruled out. Although this study was not designed to elucidate the mechanism of the kinetic interaction between G-CSF and the antibiotics, it may be speculated that, in addition to the possible effect of neutrophil recovery, significant physiological and/or haemodynamic changes may have been produced by the high G-CSF doses, with a resultant change in the disposition of the antibiotic. Caution should be exercised in directly extrapolating the observed G-CSF-mediated alteration of gentamicin kinetics to clinical situations, since the dose of the cytokine may influence the outcome of the interaction. The finding that the disposition of vancomycin was not affected by G-CSF is similar to our earlier observation that another cytokine, IFN-$\gamma$, did not affect the kinetics of vancomycin.\textsuperscript{2}

In summary, this study suggests that the outcome of GVRE infection in neutropenic hosts treated with gentamicin and vancomycin can be significantly improved by concurrent therapy with G-CSF. This is the first study to demonstrate that \textit{E. faecalis} is also susceptible to G-CSF-induced microbicidal activity. The results also corroborate the assertion that G-CSF may be beneficial as adjunctive therapy for treatment of serious microbial infections. Our findings point to the potential clinical utility of G-CSF as an adjunct to vancomycin and gentamicin for the treatment of MDRE infections in neutropenic patients.

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References


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