Concentration-dependent effects of antimicrobials on Staphylococcus aureus toxin-mediated cytokine production from peripheral blood mononuclear cells

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Background: Toxins contribute to the pathogenicity of Staphylococcus aureus infections by inducing a dysregulated inflammatory response. This study evaluated the impact of anti-staphylococcal antibiotic exposures over an increasing concentration range on cytokine production from peripheral blood mononuclear cells (PBMCs) after S. aureus toxin exposures.

Methods: Human PBMCs were suspended in complete Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum at 10^6 cells/mL with 100 ng/mL S. aureus toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxin A (SEA), α-toxin or Panton–Valentine leucocidin (PVL). Vancomycin, trimethoprim/sulfamethoxazole, tigecycline, daptomycin, linezolid, clindamycin and azithromycin were added at a concentration range of 0.5–100 mg/L. Cytokine [interleukin-1β (IL-1β), IL-6, IL-8, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α)] concentrations were measured in duplicate by ELISA following exposure and were compared with response with toxin alone.

Results: At concentrations approximating serum Cmax, tigecycline decreased IL-6 by 52%–57% and IFN-γ production by 43%–53% compared with toxin alone (P < 0.05) and linezolid inhibited TNF-α by 12%–35% and IL-8 by 25%–42% (P < 0.02). However, trimethoprim/sulfamethoxazole increased TNF-α and IL-8 production (P = 0.002). Clindamycin, daptomycin, vancomycin and azithromycin had no consistent significant effect at approximate serum Cmax concentrations. All antibiotics had a concentration-dependent effect on cytokine production, with tigecycline, clindamycin and trimethoprim/sulfamethoxazole being the most potent inhibitors of cytokine production at concentrations exceeding 25 mg/L.

Conclusions: S. aureus toxins stimulate production of inflammatory cytokines in PBMCs. Antimicrobials with high tissue penetration, including tigecycline, clindamycin, trimethoprim/sulfamethoxazole and linezolid, reduced cytokine production, which, along with their antimicrobial effects, may have importance in the therapeutic outcome of severe infections.

Keywords: community-associated MRSA, immune response, ELISA, Panton–Valentine leucocidin, monocytes

Introduction

Staphylococcus aureus is a primary cause of skin and soft tissue infections and is a prominent contributor in bloodstream, respiratory and other invasive diseases.1,2 The rapid spread of community-associated methicillin-resistant S. aureus (MRSA) is concerning due to multiple reports of overwhelming and tissue-destructive infections such as necrotizing fasciitis and necrotizing pneumonia.3–5 Community-associated MRSA and methicillin-susceptible S. aureus strains differ significantly from healthcare-associated S. aureus, in part because of a larger index of virulence factors.6,7 Highly virulent strains produce potent cytotoxins, such as Panton–Valentine leucocidin (PVL) and α-toxin, which induce leucocyte activation at low concentrations and apoptosis and cell lysis at higher concentrations.8,9 Superantigenic toxins such as staphylococcal enterotoxins A, B,
C (SEA, SEB, SEC) and toxic shock syndrome toxin-1 (TSST-1) produced by S. aureus in clinical infections non-specifically overstimulate T-helper cells, resulting in a highly dysregulated host response. Pro-inflammatory cytokines, interleukin-1β (IL-1β), IL-6, IL-8, interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) are among the immune mediators produced by T-helper cells in response to S. aureus.

Antibiotics may have immunomodulatory effects during infections caused by S. aureus. In vitro evaluations of antimicrobials have traditionally been used to predict potency, pharmacodynamic activity and treatment efficacy without consideration of their potential effect on the innate immune system. Nevertheless, modulation of the immune response may have therapeutic importance in managing severe infections. This study investigates the effect of anti-staphylococcal antibiotic exposures of increasing concentrations on cytokine production by human peripheral blood mononuclear cells (PBMCs) exposed to S. aureus toxins in vitro.

Methods

Antibiotics and toxins

Six antibiotics with activity against community-associated MRSA were evaluated: clindamycin, trimethoprim/sulfamethoxazole, vancomycin (RPI Corp., Mt. Prospect, IL, USA) and tigecycline (Tygacil, Wyeth, Madison, NJ, USA) were commercially purchased; and daptomycin (Cubist, Lexington, MA, USA) and linezolid (Pfizer, New York, NY, USA) were provided by the manufacturer. Azithromycin (RPI Corp.) is minimally active against community-associated MRSA, but it was used for comparison due to its recognized immune modulation effects. Purified SEA, TSST-1, PVL and α-toxin were obtained from Toxin Technologies, Inc. (Sarasota, FL, USA). The toxins were collected from clinical S. aureus strains: SEA from S. aureus FRI-722, TSST-1 from FRI-1169, α-toxin from Wood 46 and PVL from ATCC 49775. The highly purified toxins were endotoxin reduced to <5 endotoxin units (EU)/mg of purified toxin.

Study participants

Ten study participants were recruited from the Madison, Wisconsin, community. Healthy individuals that were eligible to participate presented with no chronic conditions or acute illnesses. Included subjects had no visible signs of skin and soft tissue infections or other inflammatory processes and were not taking any medications or dietary supplements. Healthy volunteers were chosen with no history of recent staphylococcal infections and were not taking any medications or dietary supplements. Healthy individuals that were eligible to participate presented with no visible signs of skin and soft tissue infections or other inflammatory processes and were not taking any medications or dietary supplements. Healthy volunteers were chosen with no history of recent staphylococcal infections and were not taking any medications or dietary supplements. Healthy individuals that were eligible to participate presented with no visible signs of skin and soft tissue infections or other inflammatory processes and were not taking any medications or dietary supplements. Healthy volunteers were chosen with no history of recent staphylococcal infections and were not taking any medications or dietary supplements. Healthy individuals that were eligible to participate presented with no visible signs of skin and soft tissue infections or other inflammatory processes and were not taking any medications or dietary supplements. Healthy volunteers were chosen with no history of recent staphylococcal infections and were not taking any medications or dietary supplements.

PBMC culture

PBMCs from each volunteer were cultured in complete Roswell Park Memorial Institute (RPMI) 1640 medium with 10% fetal bovine serum for 48 h at 37°C with 5% CO₂. SEA, TSST-1, PVL and α-toxin were added at a single concentration of 100 ng/mL to the 48 h culture of PBMCs. Antibiotics were added simultaneously with toxin to the culture and evaluated after 24 h as follows: first we tested the effect of antibiotics on cytokine production alone and with toxin at approximate clinical free (f) serum concentrations with 1 mg/L azithromycin, 5 mg/L clindamycin, 5 mg/L daptomycin, 25 mg/L linezolid, 1 mg/L tigecycline, 1/34 mg/L trimethoprim/sulfamethoxazole and 25 mg/L vancomycin. Second, for analysis of antibiotic concentration-dependent effects on immune response, we measured cytokine production with all antibiotics at 5, 25, 50 and 100 mg/mL alone and with toxin. All antibiotic and toxin exposures were performed in duplicate. Phytohaemagglutinin (PHA), 2.5 mg/mL, was used as a positive control. PBMCs were cultured without toxin stimulus for the negative control to determine baseline cytokine concentrations. Although purified toxins possessed low endotoxin content, PBMCs were exposed to lipopolysaccharide alone at the maximum potential concentration in the sample (5 EU/mg) to rule out endotoxin influence on the results.

Toxin cytotoxicity in PBMCs

Viable PBMCs were determined by flow cytometry. After incubation with each toxin for 24 h, the viability of PBMCs was assessed by the uptake of propidium iodide (PI). After 10 min of PI exposure, monocytes and lymphocytes were analysed by flow cytometry. A fraction of live cells in the toxin-exposed samples were compared with a fraction of live cells in the negative control (no toxin) to determine percent viability.

ELISA cytokine analysis

IL-1β, IL-6, IL-8, TNF-α and IFN-γ concentrations were measured from the supernatant of the PBMCs in cell culture with (i) exposure to the four toxins individually and (ii) in the presence of the seven antibiotics individually by ELISA (OptEIA Sets, Pharmingen, San Diego, CA, USA) according to the manufacturer’s instructions (coefficient of variation ≤10%). ELISA experiments were done in duplicate and readings were taken at 450 and 570 nm using SkanIt software for Thermo Multiskan Spectrum microplate readers (version 2.4.4). A standard curve was generated by plotting the log of the concentration of the standards versus the measured absorbance. If any absorbance reading was outside the absorbance of the highest standard concentration, the supernatant was diluted and re-assayed. Any sample with a concentration below the level of detection was assigned a value of 1 pg/mL.

Statistical analysis

Average cytokine production in PBMCs from the 10 blood donors was calculated for each antibiotic exposure. We compared the difference among cytokine concentrations in supernatant from PBMCs exposed to the toxins by analysis of variance (ANOVA) and the mean cytokine production with antibiotics to toxin control (no antibiotic) by Student’s t-test. A P value of ≤0.05 was considered statistically significant. Linear regression was used to determine the concentration-dependent effect of antibiotics on the production of cytokines from PBMCs upon toxin exposure. A probability density function for the t-test distribution ≤0.05 determined a significant concentration-dependent effect for each antibiotic versus each toxin. All statistical tests were performed using SPSS Statistical Software (Release 17, SPSS, Inc., Chicago, IL, USA).

Results

Immune response to S. aureus toxins

The relative effects of each toxin exposure on cytokine production from PBMCs were compared (Figure 1). All four toxins—PVL, TSST-1, SEA and α-toxin—at 100 ng/mL stimulated cytokine production in PBMCs. To validate the toxin concentration in this analysis, we also tested cytokine production at toxin

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concentrations of 1 ng/mL, which stimulated no cytokine production above baseline levels, and concentrations of 1000 ng/mL, which resulted in cell lysis (data not shown). When comparing within each cytokine type, we observed that PVL stimulated the highest production of TNF-α (1979 ± 1236 pg/mL) and IL-8 (322 ± 203 ng/mL), while the highest production of IL-6, IFN-γ and IL-1β was induced by the enterotoxins TSST-1 and SEA, but these were not statistically different. α-Toxin exposure induced the lowest production of cytokines TNF-α, IL-1β and IFN-γ (P < 0.001). The viability of PBMCs after 100 ng/mL toxin exposures was 91% with PVL, 96% with TSST-1, 97% with SEA and 86% with α-toxin, therefore changes in cytokine production were not due to significant decreases in monocyte or lymphocyte number. Endotoxin exposure of 5 EU/mg (the upper limit of lipopolysaccharide in the purified staphylococcal toxin) stimulated cytokine concentrations similar to the negative control and therefore had no significant impact on the results. In addition, cytokine responses in PBMCs in culture with antibiotics only (no toxin) were similar to the negative control. PHA-exposed cells had measured cytokine concentrations similar to those stimulated by toxins. Overall, the superantigen toxins (TSST-1 and SEA) were more potent stimulators of inflammatory cytokines than the cytolytic toxins (PVL and α-toxin) in PBMCs.

Comparative effects of antibiotic exposure on immune modulation

Next we compared the effects of the seven antibiotics at clinical free serum peak concentrations (Cmax) on the production of cytokines from PBMCs after exposure to the four toxins individually compared with toxin alone (Figure 2a–d and Figures S1 and S2, available as Supplementary data at JAC Online). The Cmax concentrations tested were compared with toxin control, and included vancomycin, 25 mg/L; linezolid, 25 mg/L; clindamycin, 5 mg/L; tigecycline, 1 mg/L; daptomycin, 5 mg/L; trimethoprim/sulfamethoxazole, 1/34 mg/L; and azithromycin, 5 mg/L.

Linezolid at 25 mg/L was the most effective antibiotic in reducing TNF-α and IL-8 production overall. TNF-α concentrations with linezolid decreased by 35%, 12%, 24% and 36%, and IL-8 concentrations decreased by 25%, 32%, 35% and 42% after exposure to PVL, TSST-1, SEA and α-toxin, respectively (P < 0.05). The production of IL-1β was the lowest with 1/34 mg/L trimethoprim/sulfamethoxazole, which reduced the production of this cytokine by 68%, 67% and 42% after exposure to TSST-1, SEA and α-toxin, respectively. Tigecycline at 1 mg/L inhibited cytokine response to the four toxins; notably, it was the most potent antibiotic for decreasing IL-6 (52%–57% decrease in toxin-stimulated IL-6 production) and IFN-γ (43%–53% decrease) (P < 0.05). Azithromycin (5 mg/L), clindamycin (5 mg/L), daptomycin (5 mg/L) and vancomycin (25 mg/L) each frequently reduced cytokine production from toxin exposures, but these effects were variable and not consistently significant compared with control. Among these four antibiotics, azithromycin was the most effective inhibitor of IL-1β, IL-6, IL-8 and IFN-γ with any toxin exposure (Figures S1 and S2).

The pharmacodynamic properties vary among the antibiotics evaluated in this study. This includes total drug concentrations as well as those achieved in tissues, which may be higher than those achieved in serum with repeated dosing. When comparing the antibiotic effects at high concentrations (at least 50 mg/L) on cytokine production, all antibiotics tended to reduce the production of cytokines compared with toxin alone. The most potent of these effects occurred with tigecycline, trimethoprim/sulfamethoxazole and clindamycin. After toxin exposure, 50 mg/L tigecycline reduced TNF-α production from the PBMCs by 94%–98%, IL-8 by 65%–94%, IL-6 by 64%–91% and IFN-γ by 83%–98% (P < 0.001), while trimethoprim/sulfamethoxazole reduced TNF-α production by 97%–98%, IL-8 by 78%–95%, IL-6 by 33%–83% and IFN-γ by 82%–91% (P < 0.001) (Table S1, available as Supplementary data at JAC Online). Linezolid and azithromycin effects on cytokine production at 50 mg/L were variable, but these antibiotics often reduced cytokine production. Variable effects in cytokine production overall were noted for vancomycin and daptomycin exposures at high concentrations.

Antibiotic concentration-dependent effect

Antibiotic concentrations at the infection site may be affected by tissue penetration, inflammation, patient volume and variable dosing regimens, therefore we used linear regression to establish...
the antibiotic concentration-dependent effect on cytokine production over a range of 5–100 mg/L for each antibiotic. Figure 3 displays all antibiotic concentration-dependent effects on TNF-α production from PVL toxin and IFN-γ production from TSST-1 toxin. The greatest concentration-dependent effect occurred with trimethoprim/sulfamethoxazole followed by tigecycline and clindamycin over the 5–100 mg/L antibiotic concentration range (P < 0.001). At the highest antibiotic concentration tested, 100 mg/L, these antibiotics were potent suppressors of cytokine production. Azithromycin and linezolid had lower, but still significant, concentration-dependent effects on cytokine production (P < 0.05), followed by vancomycin and daptomycin with no significant concentration effect.

Discussion

Several studies have indicated the increased virulence potential of community-associated S. aureus compared with healthcare-associated strains. After initial reports of necrotizing infections from strains with multiple virulence genes encoding for cytolytic and superantigen toxins, animal models have confirmed the role of these virulence factors in the pathogenesis of community-associated MRSA. Besides PVL, recent evidence suggests that additional virulence factors, including enterotoxins and α-toxin, are important in the inflammatory process of these infections. This study investigated the influence of antibiotics on cytokine production by PBMCs upon exposure to S. aureus toxins.

A noticeable effect was detected on PBMC production of cytokines by antibiotics at concentrations approximating those in vivo during therapy.

All toxin exposures vigorously stimulated cytokine production, but α-toxin induced significantly lower cytokine production compared with PVL, TSST-1 and SEA. In contrast to our findings, α-toxin may be a more potent stimulator of inflammatory mediators from other host cells. Bartlett and colleagues reported that the ability of α-toxin to elicit chemokines, and ultimately neutrophil recruitment, was essential for lethality in a mouse pneumonia model. Clinical strains of S. aureus are known to have heterogeneity in virulence due to polymorphisms in toxins or their regulatory genes. Since the toxins used in our study were purified from clinical strains, it may explain some of the differences in the cytokine responses in our experiments. It should also be noted that although PVL binds to monocytes and lymphocytes, it is not cytotoxic to these cells. Therefore, this study is limited in its ability to determine the complete interactions of the pore-forming toxins PVL and α-toxin, which are more relevant to neutrophil and macrophage cytotoxicity.

In our study, we provide a robust analysis of the immunomodulatory properties of available antibiotics against S. aureus. Antibiotic tissue penetration and extravascular fluid concentrations can be highly variable, but total drug concentrations approaching or exceeding those in serum have been reported in secondary compartments for tigecycline, clindamycin, trimethoprim/sulfamethoxazole and linezolid (ratio of tissue to serum reported as up to 38, 1, 1 and 8, respectively).
Therefore we examined a range of antibiotic concentrations approximating and exceeding free drug concentrations in serum and tissues with standard dosing. Using this approach, we demonstrated these agents with good tissue penetration significantly inhibited cytokine production by PBMCs with toxin exposure. We predict that modulation of the immune response may occur with these antibiotics at the site of sequestered infections such as pneumonia and deep-seated abscesses. Animal studies of these infection types could provide further histopathological evidence of reduced inflammation in S. aureus pathogenic models.

TNF-α and IL-1β, among other cytokines, are elevated in conditions of acute inflammation, such as septic shock, that have high mortality rates. This immune dysregulation may predispose patients to overwhelming infection and inflammation. We hypothesized from previous studies that protein synthesis inhibitors would have inhibitory effects on cytokine production. In our study, clindamycin had significant concentration-dependent immunomodulatory effects from 5 to 100 mg/L, with the most potent effect in decreasing IFN-γ production after PBMC exposure to PVL. Linezolid also reduced cytokine production in a concentration-dependent manner, albeit to a slightly lesser extent than clindamycin. Our results are consistent with recent data from Lambers et al. demonstrating that linezolid significantly reduces mRNA expression of IL-6, IL-8 and TNF-α, but only partially influences cytokine production. Clindamycin and linezolid overall had similar immune modulation effects to azithromycin, which is occasionally used clinically to reduce inflammation in the setting of cystic fibrosis and chronic obstructive pulmonary disease (COPD). In a related effect, Dumitrescu et al. reported that clindamycin and linezolid at subinhibitory concentrations suppress PVL production. The immunomodulatory effects of clindamycin and linezolid in our study combined with previously reported toxin inhibitory properties may offer

![Figure 3. Antibiotic concentration-dependent effect of clindamycin (CL), tigecycline (TI), trimethoprim/sulfamethoxazole (T/S), azithromycin (AZ), vancomycin (VA), linezolid (LI) and daptomycin (DA) on the in vitro production of (a) TNF-α after PVL toxin exposure and (b) IFN-γ after TSST-1 toxin exposure (n=10 donors). *P<0.001 over the concentration range for each antibiotic.](https://academic.oup.com/jac/article-abstract/67/1/123/725827)
therapeutic potential beyond antimicrobial activity in highly aggressive S. aureus infections.

Tigecycline had the most potent inhibitory effect on inflammatory cytokines. It is possible that potent inhibition of the inflammatory response may have detrimental effects on outcomes in patients with severe infections. Recently, a tigecycline labelling change by the US FDA warns of an increased risk of mortality versus comparator therapy for the treatment of severe infections.46 In a pooled analysis of clinical trials, patients with a variety of severe infections, including hospital- and ventilator-associated pneumonia, complicated skin and skin structure infections and intra-abdominal infections, had an increased risk of mortality with tigecycline therapy, although it is important to note that the mortality increase was largely due to data from the nosocomial- and ventilator-associated pneumonia patient groups.47 We raise the possibility that immune dysregulation caused by tigecycline, in particular significant inhibition of pro-inflammatory cytokines, may partially contribute to this finding. We have preliminary evidence from a clinical study of immune responses in S. aureus bacteraemia that identify elevated concentrations of IL-10, the predominant anti-inflammatory cytokine, as a significant predictor of patient mortality.48 Others have acknowledged that low inflammatory response in septic shock may result in ‘immuno-paralysis’ and lead to the development of chronic infections.49 Immune dysregulation can be triggered by multiple exogenous factors, including toxins and drugs, which impair the function of the innate immune responses of lymphocytes, monocytes and phagocytes. In cases of severe sepsis, recovery of appropriate immune function is key to survival.49 Tigecycline may alter the pro-inflammatory/anti-inflammatory cytokine balance in patients with severe infections and contribute to this observed increased mortality. Whether the significant inhibition of pro-inflammatory cytokines by tigecycline observed in this study has a direct effect on patient outcome in infections with S. aureus or other pathogens requires further clinical study.

This examination of immunomodulatory activity of antibiotics provides new insights into these properties for older antibiotics such as vancomycin and trimethoprim/sulfamethoxazole, among others, as well as newer treatment options such as daptomycin, linezolid and tigecycline. This study is limited in that it takes a one-dimensional examination of the immune response and therefore does not account for the variability of the host response in different host tissues and other cells in the immune response. As a result, only PBMCs were examined and downstream effects of these cytokine changes on other components of the innate host response were not studied. These findings indicate that antibiotic modulation of cytokine production may be a potential area for further study to improve patient outcomes in severe S. aureus infections, particularly in those mediated by pathogen toxin production.

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Transparency declarations

M. S. H. has received speaker honoraria from Merck Vaccines. G. S. has received grant funding from Cubist, is a consultant for Cubist and Astellas, and has received speaker honoraria from Cubist, Astellas and Pfizer. W. E. R. has received grant funding from Cubist and Astellas, is a consultant for Astellas and The Medicines Company, and has received speaker honoraria from Cubist. All other authors have no conflicts to declare.

Supplementary data

Figures S1, Figure S2 and Table S1 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org).

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