Virulence-Suppressing Effects of Linezolid on Methicillin-Resistant Staphylococcus aureus: Possible Contribution to Early Defervescence

Sadako Yoshizawa, Kazuhiro Tateda, Tomoo Saga, Yoshikazu Ishii, and Keizo Yamaguchi

Department of Infection Control, Toho University Omori Medical Center, Tokyo, Japan, and Department of Microbiology and Infectious Diseases, Toho University School of Medicine, Tokyo, Japan

In the present study, immunomodulatory effects of linezolid (LZD) on methicillin-resistant Staphylococcus aureus (MRSA) infections were evaluated. We have retrospectively reviewed treatment effects of LZD on 52 patients with severe MRSA infections. Sixty-four percent of the febrile patients demonstrated significant defervescence within 3 days, despite the presence of positive culture results. We speculated that this finding might be due to early anti-inflammatory effects of LZD, and to investigate this further we initiated in vivo experiments using mice MRSA pneumonia models. Mice were treated with either LZD or vancomycin (VCM) immediately after intranasal administration of MRSA. Bacterial numbers and levels of inflammatory cytokines in the lungs were determined. Although the bacterial burden in the lungs was not apparently different between the two groups, LZD but not VCM treatment significantly reduced induction of inflammatory cytokines in the lungs (P < 0.05). To evaluate whether this anti-inflammatory response was due to suppression of virulence factor expression, filter-sterilized supernatants of MRSA incubated in broth overnight with sub-MICs of LZD were subcutaneously administered to mice. To clarify whether LZD possesses direct host-modulating activity, cytokine responses to the supernatants were examined in mice pretreated with LZD. Interestingly, MRSA solutions prepared in the presence of sub-MICs of LZD revealed significant suppression of interleukin 6 (IL-6) in a dose-dependent manner (P < 0.05), but pretreatment of mice with LZD revealed no changes in cytokines. These findings suggest that sub-MICs of LZD might suppress virulence factors of MRSA, which may be associated with a reduction in endogenous pyrogens. These data may explain at least in part early defervescence observed in LZD-treated individuals.

Linezolid (LZD) (Zyvox; Pfizer, Japan) is the first available agent in a new class of antimicrobials called oxazolidinones and was approved in Japan in 2006 for treatment of methicillin-resistant Staphylococcus aureus (MRSA) infections, such as sepsis, skin and soft tissue infections, surgical site infections, and pneumonia. It has a unique mechanism of action, which is inhibition of bacterial protein synthesis at the initiation step of protein biosynthesis (24), resulting in good efficacy in treating Gram-positive bacterial infections.

Recently, several classes of antimicrobial agents, such as macrolides and quinolones, are reported to possess certain immunomodulatory effects (6, 7, 11, 16, 22, 29). In particular, protein synthesis-suppressing antibiotics, such as clindamycin and macrolides, can induce a general inhibition of virulence factor expression, such as alpha-toxin (14, 17, 21, 23, 32). Although several investigators have reported immunomodulatory activity of LZD (4, 5, 8, 9), the precise mechanism and its contribution to the clinical course are poorly understood.

After approval of LZD for MRSA infections, administration of LZD has been used generally to treat patients with severe MRSA infections. A retrospective review of data from 52 patients with severe MRSA infections treated with LZD revealed that 64% of febrile patients demonstrated significant defervescence within 3 days (defined as more than 1°C/1.8°F reduction of temperature), despite the presence of positive cultures from sterile sites. These findings prompted us to examine the effects of LZD on endogenous pyrogens in the setting of MRSA infection by utilizing mouse lung infection/sepsis models.

MATERIALS AND METHODS

Data collection from patients. A total of 52 patients with sepsis (18) due to MRSA infection and treated by LZD from January 2004 until April 2009 were eligible for analysis. A retrospective review of the treatment effects of LZD for febrile patients was performed. Febrile patients were defined as patients with fever more than 38°C (100°F) and selected from 52 patients. Times to negative culture results and defervescence were compared. This was defined as significant defervescence if the temperature was reduced more than 1°C/1.8°F.

Mice. Female BALB/c mice, 6 weeks old (Charles River Japan Inc., Yokohama, Japan) were used in these studies.

Bacteria. A clinical isolate of MRSA, toxic shock syndrome toxin 1 (TSST-1) positive and Panton-Valentine leukocidin (PVL) negative, obtained originally from blood culture in Toho University Oomori Hospital, was used throughout this study. Bacteria were stored as a 20% glycerol stock at −80°C. Bacteria were cultured from glycerol stock on 5% sheep blood agar (Mogi Corporation, Tokyo, Japan) at 37°C overnight. The bacterial suspension was prepared from the blood agar immingling with normal saline solution, adjusted as a McFarland standard of 2 (approximately 10^8 CFU/ml).

MIC determination. MIC in Mueller-Hinton (MH) broth was determined by the broth microdilution method according to CLSI standard method M100-S19 (2009). The MICs of LZD and VCM for the MRSA strain were 2.0 mg/liter and ≤1.0 mg/liter, respectively. LZD was kindly supplied by Pfizer Japan Inc., Tokyo, Japan.

Addition of bacteria and antimicrobial agents. LZD (0.4 mg/mouse; 12 mg/kg of body weight) or vancomycin (VCM) (Shionogi & Co., Ltd., Osaka, Japan) (1 mg/mouse; 40 mg/kg) was subcutaneously (s.c.) admin-
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Effects of LZD and VCM on MRSA in the lungs. To observe and compare bacterial killing effects of LZD and VCM, both were s.c. administered to mice immediately after intranasal inoculation of MRSA suspensions. Bacterial numbers in the lungs at 2 and 6 h after MRSA inoculation revealed no significant differences between LZD and VCM (Fig. 2).

LZD inhibits production of inflammatory cytokines induced by MRSA lung infection. To observe whether LZD reduces inflammatory cytokines induced by MRSA infection, cytokine levels in the lungs after MRSA inoculation were determined. IL-6, IL-12, and TNF-α levels in the lungs were significantly reduced by LZD administration but not by VCM (Fig. 3A, B, and C). Inflammatory cytokines in the cardiac blood were undetectable. Although IL-1β was secreted in both the lungs and the cardiac blood at 6 h, no suppression was observed by antimicrobial administration. IL-10 was not significantly secreted in MRSA infection (data not shown).

Inhibition of TNF-α production by LZD administration in a dose-dependent manner. To confirm the dose dependency of these phenomena, LZD was administered as 40, 20, and 10 mg/kg (1, 0.5, and 0.25 mg/mouse, respectively) immediately after MRSA administration, and VCM was administered as 40 mg/kg (1 mg/mouse) (n = 7/group). TNF-α and IL-6 in the lungs were significantly reduced by LZD administration in a dose-dependent manner but not by VCM (Fig. 4A and B). Bacterial numbers in the lungs revealed no significant differences between the LZD- and VCM-administered groups (data not shown).

Sub-MICs of LZD reduced virulence factor production in MRSA. To explore the mechanisms of suppression of inflammatory cytokines in the lungs of mice treated with LZD, the following experiments were performed. LZD was diluted to several sub-MICs and incubated overnight by constant shaking with MRSA to evaluate whether LZD suppresses virulence factor production of MRSA. The overnight solution was filter sterilized and administered to the mice. On the other hand, to confirm modulation of host inflammatory responses, LZD was administered 1 h prior to the administration of filter-sterilized MRSA growth medium, which was grown in the absence of LZD. Serum inflammatory cytokine levels were determined 2 h after LZD administration. To exclude the possibility of reduced production of inflammatory cytokines due to antibacterial effects by LZD, the bacterial number of MRSA under each condition was determined. Although bacterial numbers were not significantly different between the groups

RESULTS

Early defervescence was observed among LZD-treated patients even in a culture-positive condition. In the course of treating MRSA-infected septic patients with LZD, it was observed that patients treated with LZD tended to defervescence promptly. To investigate this further, a retrospective review of the effects of LZD on 52 patients with MRSA infections was undertaken. Sixty-four percent of febrile patients demonstrated significant defervescence within 3 days despite having positive culture results. The median time to negative culture results was 8 days, and defervescence was significantly earlier than that with the culture-negative condition (P < 0.001, Fig. 1).

FIG 1 Comparison of times to defervescence and negative culture results among MRSA septic patients. Among 52 patients with severe MRSA infection treated by LZD, 28 patients revealed apparent fever. They were retrospectively reviewed, and times to defervescence and negative culture results were compared. Defervescence was defined as a more than 1°C/1.8°F reduction of temperature. A culture specimen was obtained almost twice a week. The first day of LZD administration was counted as day 1.

FIG 2 Bacterial numbers in the lungs after MRSA nasal inoculation. MRSA was intranasally inoculated into BALB/c mice, and antimicrobial agents were subcutaneously administered immediately after inoculation. Bacterial numbers in the lungs at 2 and 6 h after MRSA inoculation revealed no significant differences between LZD and VCM (Fig. 2).

CFU determinations and cytokine analysis. Mice were sacrificed by CO2 asphyxia, and lung homogenate and cardiac blood at 2 and 6 h after infection were used for bacterial quantification and measurement of inflammatory cytokines (interleukin 1β [IL-1β], IL-6, IL-10, IL-12, and tumor necrosis factor alpha [TNF-α]) production. Bacterial numbers were quantified by plating serial saline dilutions of samples onto 5% sheep blood agar, followed by overnight incubation.

ELISAs for cytokines. Inflammatory cytokine levels were determined by sandwich ELISAs using antibody pairs from R&D Systems, Inc., as per the manufacturer’s instructions. Model experiments were performed in duplicate to ensure reproducibility.

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of 0.5 and 0.25 μg/ml of LZD and the control (Fig. 5), IL-6 induced by MRSA infection was significantly suppressed by sub-MICs of LZD. In contrast, prior administration of LZD did not suppress IL-6 production (Fig. 6). These results suggest that suppression of inflammatory cytokines by administration of LZD may be due to the inhibition of virulence factors of MRSA rather than modulation of host inflammatory responses by LZD.

**DISCUSSION**

Recently, some antimicrobial agents, such as macrolides, have been reported to possess immunomodulatory effects in addition to their antimicrobial activity. Erythromycin inhibits the production of several proinflammatory cytokines through an inhibitory effect on the activation of transcription factors, such as NF-κB, in the process of signal transduction and DNA-binding activity, which results in a lack of transcriptional activation of target genes (7). The clinical efficacies of antimicrobial agents are determined not only by their respective bactericidal or bacteriostatic activities and pharmacokinetics but also by their action on bacterial virulence factor suppression, especially at suboptimal concentrations. Protein synthesis-suppressing agents, such as clindamycin and macrolides, can induce a general inhibition of exotoxin production (14, 17, 21, 23, 32). Accumulating in vitro and in vivo studies have investigated the efficacy of clindamycin in the treatment of group A streptococcus infections (10, 20, 25–27). Stevens et al. investigated the relative efficacies of several antimicrobial agents, including clindamycin, in a mouse model of myositis due to *Strep*...
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ting models (8, 28). These studies examined immunomodula-

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These reports describe the immunomodulatory effects of LZD on


reduced the secretion of specific virulence factors, including

S. pyogenes experimental models (9). Coyle et al. demonstrated

factor expression at sub-MICs of LZD using

effects of LZD. Gemmell et al. revealed a reduction in virulence

MRSA suspension. Inflammatory cytokine levels in the cardiac blood were

investigated at 2 h after infection. *, P < 0.05 compared to results for MRSA

single infection; †, P < 0.01 compared to results for MRSA plus LZD 1 y. Tx, treatment.

tococcus pyogenes (25). They suggested that the enhanced efficacy of clindamycin could be related to several inherent properties, including the inhibition of S. pyogenes virulence factors, particularly the M protein (25). Furthermore, Gemmell et al. have determined that in the presence of various concentrations of clindamy-

cin and LZD, enhanced opsonization and phagocytosis of S. pyogenes are seen (9, 10).

LZD is a member of the new synthetic class of antibacterial oxazolidinones that inhibit bacterial protein synthesis at the initi-

ation step of protein biosynthesis (24). Although its efficacy is well

known and the molecular mode of action has been determined, little information was available about the immunomodulatory ef-

fects of LZD. Gemmell et al. revealed a reduction in virulence factor expression at sub-MICs of LZD using in vitro S. aureus and S. pyogenes experimental models (9). Coyle et al. demonstrated that some antimicrobial agents, including LZD, reduced exotoxin release (5). Furthermore, Bernardo et al. demonstrated that LZD reduced the secretion of specific virulence factors, including staphylococcal enterotoxin A (SEA) and SEB, bifunctional auto-

lysin, autolysin, protein A, and alpha- and beta-hemolysins (4). These reports describe the immunomodulatory effects of LZD on bacteria. On the other hand, Garcia-Roca et al. and Takahashi et al. demonstrated reduced cytokine synthesis from peripheral blood cells induced by LPS stimulation, also using in vitro experimental models (8, 28). These studies examined immunomodu-

latory effects of LZD in the host.

In the present study, first of all, we noticed an impressive phe-
nomenon during treatment of MRSA septic patients with LZD. Febrile patients treated with LZD promptly achieved deferves-
cence despite being culture positive. Then, we considered possible immunomodulatory effects of these antimicrobial agents and per-
formed in vivo experiment using an MRSA lung infection model. Impressively, TNF-α, IL-6, and IL-12 were suppressed signifi-
cantly by LZD in a dose-dependent manner, but not by VCM, without showing a bacterial killing effect. Since the precise mech-

anism of possible immunomodulation by LZD has not yet been determined, we considered two hypotheses, i.e., suppression of virulence factor expression of MRSA and modulation of host in-

flammatory responses by LZD. As shown in Fig. 6, prior administra-

tion of LZD to mice did not reduce IL-6 production in chal-

lenge with filter-sterilized MRSA supernatant, whereas significant suppression of IL-6 was observed, when MRSA was incubated with sub-MICs of LZD, even at a concentration that did not affect bacterial numbers.

The immunoregulatory activities of antimicrobial agents may, in addition to their antimicrobial effects, have a protective effect against the destructive local inflammatory response in areas of infection. The present data suggest potent virulence factor-sup-

pressing activity of LZD, which results in a reduction of inflam-

matory cytokine production. Since these effects were observed at

LZD concentrations that are achievable in human serum with the con-

ventional dosing, they may explain at least in part early deferver-

cence observed in patients treated with LZD, despite the pres-

ence of positive cultures of MRSA from normally sterile sites.

The appearance and spread of antibiotic-resistant organisms, including community-acquired MRSA, in addition to an increase in incidence of refractory and life-threatening infections by staphy-

lococci, are of great concern (3, 15, 19). Since development of new compounds against antibiotic-resistant organisms is limited, it may be crucial to search for and find novel applications and additional activities of antibiotics. The present data constitute one such example, which may be associated with a clinical phenom-

enon, early defervescence, observed in patients treated with LZD.

Further investigations of molecular mechanisms of suppression of virulence of MRSA by LZD, in addition to well-planned clinical and epidemiological trials with MRSA infections, are warranted.

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