

Synergistic antimicrobial effect of cefotaxime and minocycline on proinflammatory cytokine levels in a murine model of *Vibrio vulnificus* infection

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Background and Purpose: *Vibrio vulnificus* causes primary bacteremia and necrotizing wound infection, leading to high morbidity and mortality in humans. This study aimed to evaluate the antimicrobial effect of cefotaxime and minocycline on proinflammatory cytokine levels in a murine model of *V. vulnificus* infection.

Methods: We investigated the dynamics of proinflammatory cytokines and their modulation by antimicrobial agents using a murine model of *V. vulnificus* infection. The change in cytokine levels was followed over a time course to identify the antimicrobial activity of the drugs against *V. vulnificus*. BALB/c female mice were challenged with an intraperitoneal infection using a clinical invasive isolate of Vv05191, and their cytokine levels were assayed over various time points.

Results: Serum levels of tumor necrosis factor- α , interleukin (IL)-1 β , and IL-6 post-infection were found to be inoculum dose-dependent and positively correlated to the subsequent fatality rate in the infected mice. With an inoculum of 6.6×10^6 colony-forming units and intraperitoneal administration of cefotaxime, minocycline, or both, the serum and peritoneal fluid cytokine levels increased and then declined gradually. Comparison of the 3 antimicrobial regimens revealed that the magnitude of reduction in cytokine levels was greatest in mice treated with cefotaxime-minocycline combination. Moreover, the peritoneal fluid cytokine level in the combination group was significantly lower than that in the groups treated with minocycline or cefotaxime alone.

Conclusions: The current results support the superiority of the combination therapy in treating invasive *V. vulnificus* infections.

Key words: Antibacterial agents; Cytokines; Drug therapy, combination; *Vibrio vulnificus*

Introduction

Vibrio vulnificus, an estuarine- and seawater-derived [1, 2] Gram-negative halophilic bacterium, causes primary sepsis, wound infection, and gastrointestinal illness in humans [1,3,4]. Clinically, *V. vulnificus* infection leads to fulminant sepsis, especially in cirrhotic patients, with a mortality rate of 55%. Necrotizing fasciitis, usually

resulting from wound infections, can lead to substantial morbidity and mortality [4]. Therefore, successful management of fulminant *V. vulnificus* infections requires highly active antimicrobial agents.

More than two decades ago, Morris and Tenney [5] and Morris and Black [6] had stressed the superiority of tetracycline over cefotaxime in the treatment of *V. vulnificus* infections. Jawetz and Gunnison noted that bacteriostatic antibiotics may antagonize the actions of bactericidal drugs [7]. However, we recently showed that combination therapy using cefotaxime and minocycline is more effective in the treatment of severe

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experimental *V. vulnificus* infections than monotherapy with either monocycline or cefotaxime alone, both in vitro and in animal studies [8,9].

It is well known that an excess of proinflammatory cytokines induced by Gram-negative bacteria is associated with the clinical manifestations of septic shock and increased mortality [10]. Tetracycline and its derivatives, such as minocycline, a bacteriostatic antibiotic, were noted to have not only antibacterial effects but also immunomodulatory effects [11-13]. Similarly, cefotaxime, a bactericidal antibiotic, is also a potent immunodepressing antibiotic [14,15]. There is very limited information about the antimicrobial or immunomodulatory effects of the different antibiotics on cytokine production in both patients and in animal models of *V. vulnificus* infection. Only doxycycline has been reported to decrease serum levels of tumor necrosis factor-alpha (TNF- α) and interleukin (IL)-1 beta (IL-1 β) in patients with *V. vulnificus* septicemia [16]. It is also unknown whether improved outcome of the combination of cefotaxime-minocycline over minocycline or cefotaxime alone in the treatment of *V. vulnificus* infection is related to the downregulation of cytokine levels. Therefore, we investigated the antimicrobial effects of cefotaxime and minocycline on the profiles of serum and peritoneal fluid proinflammatory cytokines using a murine model of intraperitoneal *V. vulnificus* infection.

Methods

Reagents

Lipopolysaccharide (LPS, *Escherichia coli* strain 055:85) was purchased from Sigma (St. Louis, MO, USA) and dissolved in 0.85% saline at 1 mg/mL concentration. Commercially available cefotaxime (Hoechst AG, Frankfurt, Germany) and minocycline (Lederle Parenterals, Inc., Carolina, USA) were used for the in vitro and murine experiments.

Bacterial preparation

The clinical isolate of encapsulated *V. vulnificus*, Chi-Mei Vv05191, obtained from Chi-Mei Medical Center was used for the study. The organism was stored at 70°C in Luria-Bertani broth (Difco Laboratories, Detroit, MI, USA) before being cultured on nutrient agar (Difco Laboratories) with 3% sodium chloride. Bacteria grown in the agar medium were maintained at room temperature, from which the inoculation suspension was prepared in Mueller-Hinton broth (Difco Laboratories).

The bacterial inocula were prepared as follows. The frozen stock was thawed at room temperature and grown on agar. Single colony was chosen and cultured overnight in freshly prepared Mueller-Hinton broth at 35°C. Then, 400 μ L of the bacterial suspension was diluted 1:50 in Mueller-Hinton broth and incubated under the same conditions for 4 h. Bacteria were collected by centrifugation at 10,000 rpm for 10 min at 24°C. Pellets were resuspended and diluted to a final bacterial concentration of 1×10^7 colony-forming units (CFU)/mL (optical density, 0.22-0.25) before injection into mice. The inoculum concentration was confirmed by the subsequent growth of the concurrent culture on agar plates.

Mice

Inbred BALB/c female mice (4-5 weeks old), weighing 20 g, were purchased from the Animal Center, National Science Council, Taipei, Taiwan. They were allowed to acclimatize for 5 to 7 days in the animal research laboratory of Chi-Mei Medical Center. Food and water were supplied ad libitum.

Preparation of antimicrobial agents

Antibiotic dosages were given in accordance with the values recommended by the manufacturers: 300 mg/kg of cefotaxime at 2, 5, and 8 h, 4 mg/kg of minocycline at 2 h after inoculation with Vv05191. They were tested alone and in combination. Antibiotics were dissolved in sterile Milli-Q deionized water, filtered, sterilized, and administered at 0.1 mL dosage per injection starting at 2 h after the intraperitoneal bacterial inoculation.

Preliminary study

Preliminary studies were conducted to determine the optimal inoculum dosage and appropriate timing for the sampling and quantification of serum cytokines. The Vv05191 dosages tested were 10^5 , 10^6 , and 10^7 CFU for 6 mice per group. Blood samples were obtained by incising the left axillary artery of mice at 0, 3, 6, and 9 h post-infection. Subsequent studies were performed using 10^6 CFU Vv05191 for intraperitoneal inoculation, and peritoneal fluid and serum cytokine levels were measured at 3, 6, and 9 h post-infection.

Effect of antibiotics on cytokine production from the murine macrophage cell line RAW264.7

In vitro studies were performed to differentiate between the effects of lowered cytokine levels due to antimicrobial activity or immunomodulatory mechanisms.

RAW264.7 cells were obtained from the American Type Culture Collection and maintained in Dulbecco's Modified Eagle's Medium (Biosource, Rockville, MD, USA) with 10% heat-inactivated fetal bovine serum (Hyclone Laboratories, Logan, UT, USA) at 5% carbon dioxide. The RAW264.7 cells were washed twice with phosphate-buffered saline and harvested by scraping. After a 24-h incubation period in 5% carbon dioxide, the cells were plated in 24-well plates at a density of 1×10^6 cells/mL per well and exposed to heat-treated Vv05191 at 1×10^8 CFU/mL or LPS at 1 μ g/mL. Cultures were treated with minocycline (1 μ g/mL), cefotaxime (40 μ g/mL), or both. The drug concentrations used were standard drug dosages that were previously shown to achieve peak human plasma levels after intravenous administration [17]. The cytokine levels in cultures that received no antibiotics and Vv05191 treatment were used as controls. After a 24-h incubation period, the cell-free supernatants were collected by centrifugation at 3500 rpm, and their TNF- α and IL-6 concentrations were measured at 6, 12, and 24 h after application of antibiotics and Vv05191 inoculation.

Murine model with *V. vulnificus* infection

BALB/c mice were divided into 7 groups of 10 mice each. Four control groups were used. Mice in the negative control group were injected with normal saline, cefotaxime, or minocycline instead of the *V. vulnificus* bacterial suspension. Mice in the positive control group were treated with 0.85% saline after Vv05191 infection. There were 3 antibiotic treatment groups — receiving cefotaxime or minocycline alone, or both. Saline or antibiotic treatments were administered via intraperitoneal injection 2 h after Vv05191 inoculation. Mice were sacrificed by overdose of pentobarbital before the harvesting of samples. All animal experiments were conducted in compliance with the relevant national guidelines of the Republic of China and approved by the Chi-Mei Foundation Medical Center Animal Use Policy.

Serum and peritoneal fluid levels of TNF- α , IL-1 β , and IL-6

After the antibiotic therapy, mice were sacrificed at 3, 6, and 9 h after the intraperitoneal inoculation of Vv05191. Blood samples were obtained by incising the left axillary artery of mice. Samples were kept overnight at 4°C to allow clotting. In the 3 negative control groups, blood samples were collected at 1, 4, and 7 h after the intraperitoneal injection of saline, cefotaxime,

or minocycline. The blood specimens were collected, centrifuged at 3000 rpm for 20 min at room temperature, and the resulting sera were stored at -80°C.

To obtain peritoneal fluid samples, 3 mL of 0.85% saline was injected into the peritoneal cavity of mice using a 3-mL Terumo syringe (Terumo, Tokyo, Japan) and gently massaged for 1 min. About 3 mL of peritoneal fluid was then harvested, centrifuged at 3000 rpm for 20 min at 4°C, and the supernatant was stored at -80°C.

Measurement of cytokine concentrations

To determine the concentrations of TNF- α , IL-1 β , and IL-6, commercially available enzyme-linked immunosorbent assay kits (R & D Systems, Minneapolis, MN, USA) were used in accordance with the manufacturer's instructions. The quantification of cytokine level was based on a standard curve derived by linear dilution of the cytokine standards that were included in the kit. Assays were performed in duplicate.

Statistical analysis

Data were processed and analyzed using Statistical Package for the Social Sciences (SPSS) for Windows (Version 10.0.7; SPSS Inc., Chicago, IL, USA). The Mann-Whitney *U* test was performed to test the difference in cytokine levels between the groups treated with antibiotics and the control group for every time point. Kruskal-Wallis H test was performed to test the difference in cytokine levels among the groups at 3 h; an alpha level of 0.05 was regarded as statistically significant. If significant difference was detected, post hoc multiple comparisons were then performed using the Dunn test for paired comparison. The Bonferroni rule for the correction of α level was also used; $p < 0.0083$ was considered significant.

Results

Mortality and proinflammatory cytokine levels

Cytokine levels were undetectable in the serum of mice treated with intraperitoneal administration of saline, cefotaxime, or minocycline alone at all the time points studied after the intraperitoneal injection (data not shown).

All the mice survived for at least 9 h when treated with low concentrations of viable Vv05191 (1.2×10^5 CFU). Serum TNF- α , IL-1 β , and IL-6 peaked at 3 h after infection, but rapidly declined thereafter. At 6 h post-infection, the 3 proinflammatory cytokine levels dropped to less than half of that observed at 3 h, while

Table 1. Mortality and serum proinflammatory cytokine levels of mice infected with Vv05191

Inoculum (CFU)	Time after inoculation (h)	No. of mice surviving	Mortality (%)	Serum cytokine levels in surviving mice (pg/mL) [mean \pm SD]		
				TNF- α	IL-1 β	IL-6
1.2×10^5	0	6		x	x	x
	3	6	0	84.5 \pm 18.7	174.3 \pm 42.3	2014.7 \pm 344.2
	6	6	0	41.4 \pm 7.6	64.36 \pm 16.9	663.4 \pm 102.1
	9	6	0	4.2 \pm 1.3	12.1 \pm 4.2	134.5 \pm 14.2
2.6×10^6	0	6		x	x	x
	3	6	0	654.2 \pm 102.5	330.6 \pm 54.8	5796.3 \pm 1342.1
	6	6	0	974.3 \pm 186.8	4328.5 \pm 687.4	11,223 \pm 2676.5
	9	6	0	1874.2 \pm 345.2	10,418 \pm 1245.3	20,476 \pm 3450.3
1.8×10^7	0	6		x	x	x
	3	6	0	2481.1 \pm 311.3	3619.3 \pm 966.4	33,754 \pm 4453
	6	0	6/6 (100)	-	-	-

Abbreviations: CFU = colony-forming units; SD = standard deviation; TNF- α = tumor necrosis factor-alpha; IL-1 β = interleukin-1 beta; IL-6 = interleukin-6; x = undetectable serum cytokine level

the concentration of cytokine was the lowest at 9 h (Table 1).

When the inoculum was increased to 2.6×10^6 CFU all the 3 proinflammatory cytokines in the serum gradually increased over 9 h after Vv05191 infection (Table 1). The change in serum IL-1 β level was the greatest — its level at 9 h was 31-fold that observed at 3 h.

All 6 mice died within 6 h when the inoculum of Vv05191 was increased from 2.6×10^6 CFU to a dose of 1.8×10^7 CFU; thus, serum cytokine levels were only measured at 3 h, accounting for a 4-fold increase in serum TNF- α , an 11-fold increase in IL-1 β , and a 6-fold increase in IL-6 levels. At this time point, serum TNF- α , IL-1 β , and IL-6 levels were positively correlated with the inoculum dose and with the mortality rate of the infected mice at 9 h (0% at 1.2×10^5 CFU and 100% at 1.8×10^7 CFU) [Table 1].

We concluded that an inoculum size of 10^6 was optimal for the subsequent experiments aimed to evaluate the effects of various antibiotic regimens in modulating the serum and peritoneal fluid cytokine levels.

Effect of antimicrobial agents on cytokine production from stimulated RAW264.7

To differentiate between the effects of immunomodulation and antimicrobial activity in the downregulation of cytokines, we conducted in vitro studies to determine whether cefotaxime and minocycline, alone and in combination, can depress cytokine levels. The TNF- α and IL-6 concentrations were assayed after antibiotic or LPS treatment in the control, minocycline-treated, cefotaxime-treated, and minocycline-cefotaxime-treated groups.

The trends of cytokine expression in vitro were similar in Vv05191 and LPS treatment groups, where the TNF- α and IL-6 levels increased dramatically at 6 h after 1 μ g/mL LPS and Vv05191 treatment, with the concentrations of TNF- α peaking at 6 h and IL-6 at 24 h. The 6-h TNF- α levels were 19,425, 18,033, 19,800, and 18,700 pg/mL and the 24-h IL-6 levels were 3218, 2584, 2910, and 2560 pg/mL in no antibiotic, minocycline, cefotaxime and minocycline-cefotaxime combination treatment, respectively. Also, no significant downregulation of cytokines was observed when RAW264.7 was used with the different antibiotic regimens when co-cultivated with heat-treated Vv05191 or LPS (Table 2) in the time-course study. This indicates that there was no immunomodulation effect on cytokine levels by any of the antibiotic regimens.

Modulation of serum proinflammatory cytokine levels

To study the effect of antimicrobial therapy on the cytokine profiles, the infectious dose of Vv05191 was kept at 6.6×10^6 CFU for the following experiments. All infected mice that were on antimicrobial therapy survived at least 9 h post-Vv05191 inoculation. Without antimicrobial therapy, serum TNF- α , IL-1 β , and IL-6 levels gradually increased, except for the TNF- α concentrations at 6 h which showed a slight decline from 3 h (Fig. 1a, 1b, and 1c). With antibiotic therapy, irrespective of the therapeutic regimen used, the levels of all 3 cytokines decreased dramatically until 9 h. Mean serum levels of TNF- α , IL-1 β , and IL-6 at 9 h after Vv05191 infection without therapy were 2744, 17,880, and 26,200 pg/mL, respectively. For the

Table 2. Effect of antimicrobial agents on time course of cytokine production from stimulated RAW264.7

Cytokine concentration	Treatment	Vv05191			1 µg/mL LPS			Control		
		6 h	12 h	24 h	6 h	12 h	24 h	6 h	12 h	24 h
TNF-α (pg/mL)	No antibiotic	19,425 ± 984	19,067 ± 289	15,700 ± 406	17,800 ± 762	17,600 ± 1552	12,833 ± 153	1239 ± 127	1829 ± 106	1729 + 101
	MIN (1 µg/mL)	18,033 ± 208	18,033 ± 666	14,000 ± 100	18,900 ± 917	17,167 ± 907	13,067 ± 231	1212 ± 102	1829 ± 117	1703 + 86
	CTX (40 µg/mL)	19,800 ± 436	17,467 ± 252	14,833 ± 416	18,367 ± 81	18,567 ± 808	14,067 ± 586	1293 ± 110	1773 ± 121	1717 + 92
	MIN + CTX	18,700 ± 436	17,500 ± 458	13,567 ± 252	17,867 ± 1361	17,067 ± 351	13,733 ± 115	1345 ± 94	1773 ± 30	1677 + 102
	IL-6 (pg/mL)	No antibiotic	734 ± 115	2837 ± 309	3218 ± 135	1090 ± 107	3304 ± 300	4157 ± 378	0 ^a	0
	MIN (1 µg/mL)	656 ± 89	2510 ± 89	2584 ± 233	1088 ± 37	3379 ± 340	3705 ± 157	0	0	0
	CTX (40 µg/mL)	682 ± 57	2648 ± 154	2910 ± 279	951 ± 20	3414 ± 176	4081 ± 404	0	0	0
	MIN + CTX	638 ± 15	2625 ± 223	2560 ± 305	1009 ± 68	3192 ± 138	3923 ± 160	0	0	0

Abbreviations: LPS = lipopolysaccharide; TNF-α = tumor necrosis factor-alpha; MIN = minocycline; CTX = cefotaxime; IL-6 = interleukin-6
^aIndicates without testing.

cefotaxime-minocycline combination therapy, however, the levels of TNF-α, IL-1β, and IL-6 decreased to 1.57, 4.11, and 114.68 pg/mL, respectively at 9 h. Such a change accounts for a 1748-fold reduction in serum TNF-α, 4350-fold in IL-1β, and 228-fold in IL-6 after the combined regimen, and a 398-fold reduction in TNF-α, 4009-fold in IL-1β, and 120-fold in IL-6 after minocycline treatment; however, only a 30-fold reduction in TNF-α, 41-fold in IL-1β, and 79-fold in IL-6 occurred after cefotaxime treatment.

There was no difference in the experimental outcomes among the 3 antimicrobial regimens. The trends of changes in the serum cytokine levels were similar between the cefotaxime-minocycline combined regimen and the minocycline regimen, but different with the cefotaxime regimen. The downregulation of cytokines in both the combined and minocycline regimens were significantly different from the positive control group at every given time point ($p < 0.0083$, Dunn test). The downregulation of TNF-α, IL-1β, and IL-6 was the lowest in the cefotaxime treatment group, in which the cytokine levels were not significantly different from those in the positive control group at any time point. Furthermore, at 3 h post-infection, this group did not show downregulation of cytokine levels; however, the TNF-α and IL-1β cytokine levels were slightly higher than those of mice receiving no therapy (control group),

although the difference was not statistically significant ($p > 0.0083$, Dunn test).

The combined regimen and minocycline treatment showed nearly the same effect of downregulation of the cytokine levels. The combined regimens was more efficient in lowering cytokine levels than minocycline treatment; however, there was no significant difference in cytokine levels between the 2 groups at any time point ($p > 0.0083$, Dunn test).

Modulation of the peritoneal fluid proinflammatory cytokine levels

The recovered peritoneal fluid was assayed for cytokine concentrations. Without antimicrobial therapy, the peritoneal fluid TNF-α, IL-1β, and IL-6 levels gradually increased following a similar pattern to that observed in the serum. With antimicrobial therapy, irrespective of the therapeutic regimen used, cytokine levels decreased gradually until 9 h (Fig. 2a, 2b, and 2c). The average levels of TNF-α, IL-1β, and IL-6 in the untreated mice at 9 h after Vv05191 infection were 1336.3, 13,820.0, and 22,540.0 pg/mL, respectively. In contrast, 9 h after cefotaxime-minocycline combination therapy, peritoneal fluid levels of TNF-α, IL-1β, and IL-6 decreased to 3.9, 149.3, and 143.4 pg/mL, respectively. Similarly, after minocycline treatment, the levels decreased to 42.7, 490.3, and 2570.8 pg/mL, respectively. Such changes correspond

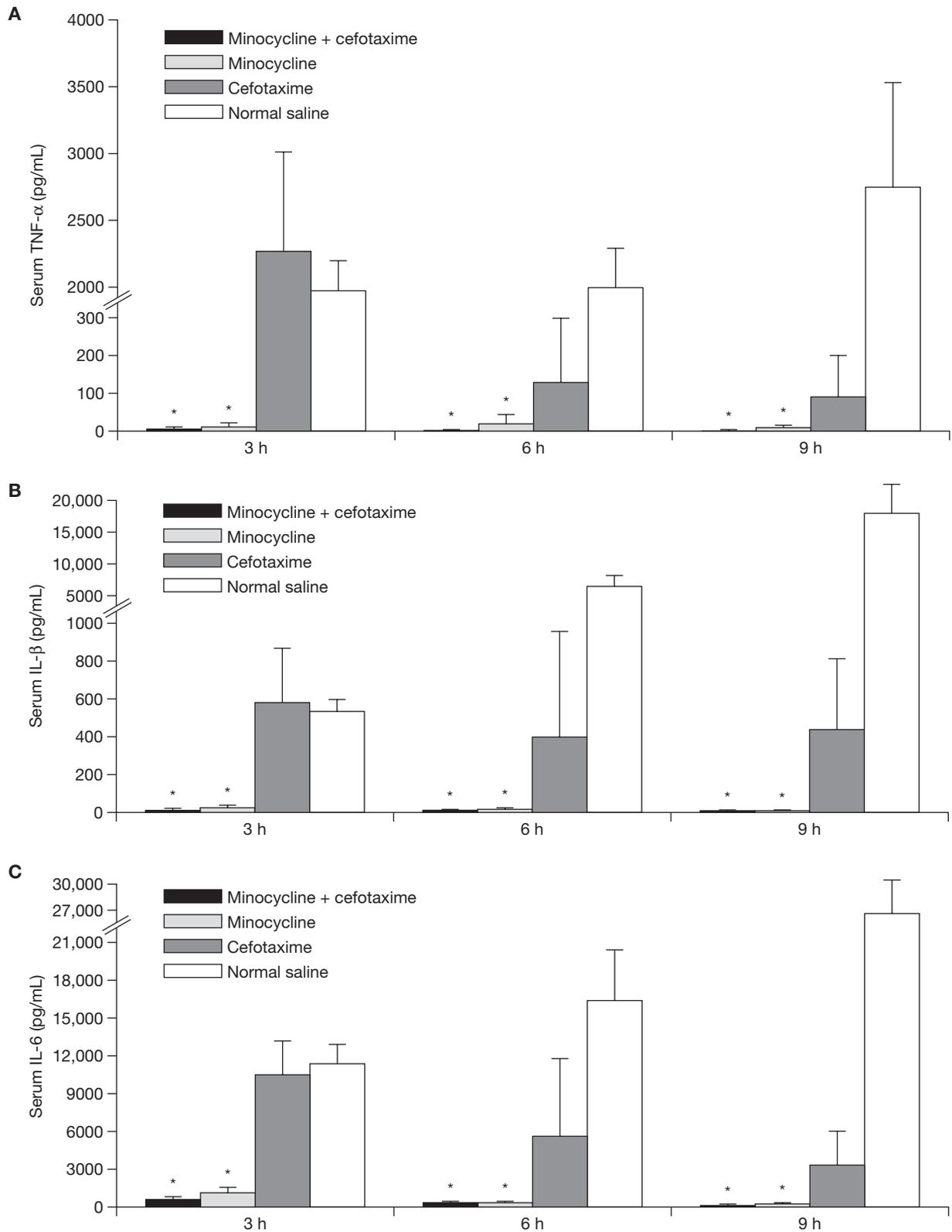


Fig. 1. Serum tumor necrosis factor-alpha (TNF- α) [A], interleukin-1 beta (IL-1 β) [B], and IL-6 (C) levels in mice treated with 3 antimicrobial regimens and normal saline (control group) after intraperitoneal inoculation of 6.6×10^6 colony-forming units of Vv05191. Bars represent standard deviation. [*] indicates serum cytokine levels in mice treated with antibiotics that are significantly lower than those in the control group ($p < 0.0083$).

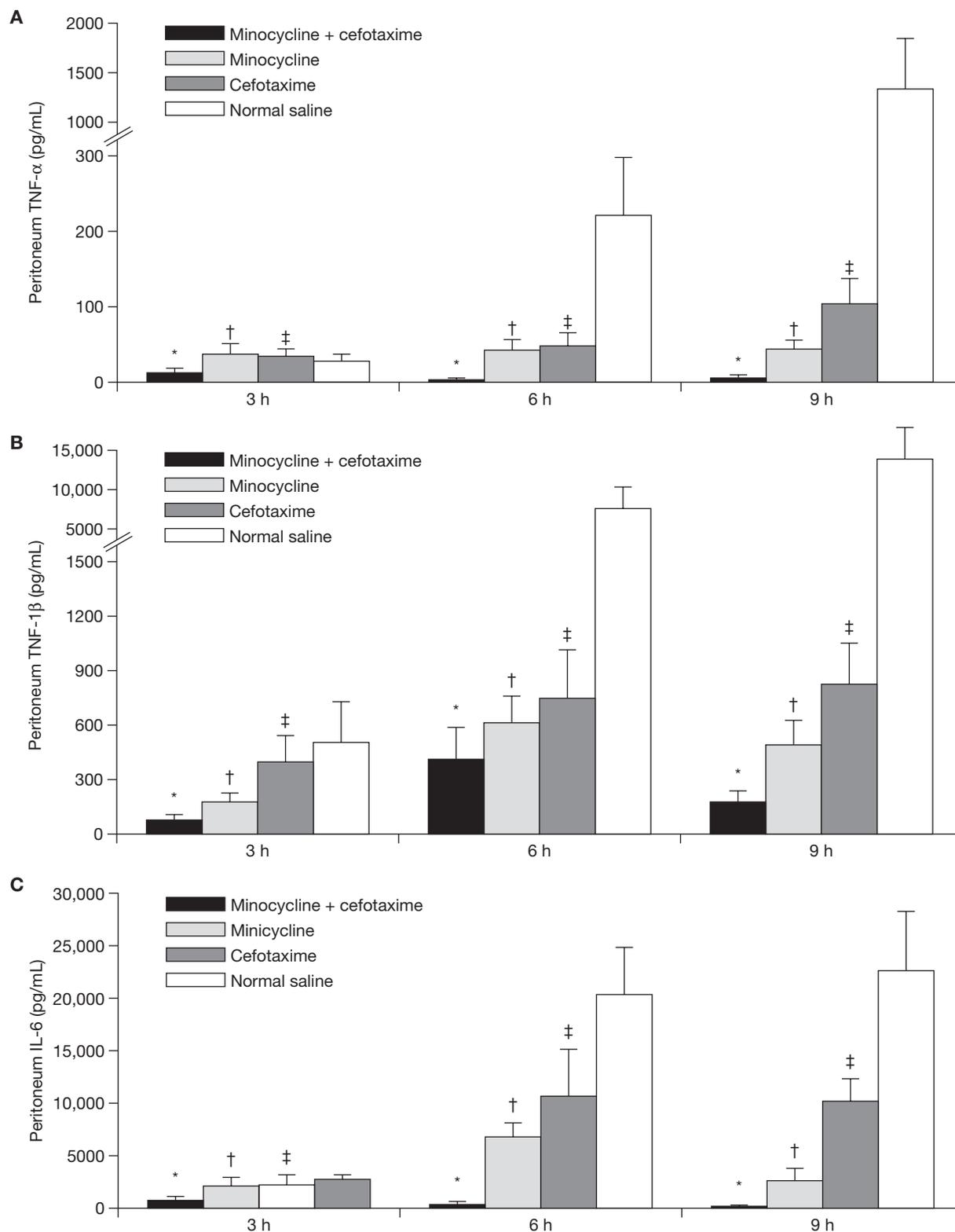


Fig. 2. Peritoneal fluid tumor necrosis factor- α (TNF- α) [A], interleukin-1 beta (IL-1 β) [B], and IL-6 (C) levels in mice treated with 3 antimicrobial regimens and normal saline (control group) after intraperitoneal inoculation of 6.6×10^6 colony-forming units of Vv05191. Bars represent standard deviation. [*] indicates cytokine levels in mice treated with antibiotics that are significantly lower than those in the control group ($p < 0.0083$). [†] and [‡] indicate cytokine levels in mice treated with combined antibiotics that are significantly lower than those in minocycline and cefotaxime treatment groups, respectively ($p < 0.0083$).

to a 342-fold reduction in serum TNF- α , 93-fold in IL-1 β , and 157-fold in IL-6 after the combined regimen, but only a 31-fold reduction in serum TNF- α , 28-fold in IL-1 β , and 8.7-fold in IL-6 in the minocycline group.

The trends of changes in the intraperitoneal cytokine levels were similar in the 3 regimen groups. However, the modifications in cytokine responses varied greatly. The downregulation of TNF- α , IL-1 β , and IL-6 released into the peritoneal fluid was greatest in the cefotaxime-minocycline treatment group, and smallest in the cefotaxime group (Fig. 2). Further, cytokine levels were only significantly lowered in the cefotaxime-minocycline group when compared to the positive control group ($p < 0.0083$, Dunn test) at all time points; moreover, their levels were also significantly lower than those in mice treated with minocycline or cefotaxime ($p < 0.0083$, Dunn test) alone, except the IL-1 β level at 6 h after inoculation in the minocycline-treated mice ($p = 0.071$, Dunn test). There is a difference in the efficiency of downregulation of cytokine levels in the peritoneal fluid between the combination therapy and minocycline regimens. Also, at 3 h, the TNF- α peritoneal fluid cytokine levels in the minocycline-treated mice were higher than those in mice receiving no antibiotic treatment, although the difference was not significant ($p > 0.0083$, Dunn test).

The modulatory effects of cefotaxime on peritoneal fluid cytokines were similar to the serum study where at 3 h the TNF- α levels were higher than those in the control group.

Discussion

Evidence from animal studies and clinical investigations has demonstrated that deregulation of the immune system and overproduction of circulating proinflammatory cytokines lead to sepsis syndrome, septic shock, and multiple organ failure [18,19]. In animal studies using large doses of bacteria or endotoxin, high titers of proinflammatory cytokines, especially TNF- α , are observed and correspond to a poor prognosis [20,21]. Such immunopathological alterations were also found in early clinical studies involving children with meningococemia, where the high levels of circulating TNF- α were correlated with increased fatality [22,23]. It is not surprising that *V. vulnificus* infections in humans, characterized by primary bacteremia or severe necrotizing soft tissue infections can result in similar overexpression of immune changes.

Shin and co-workers [16] reported that the levels of the proinflammatory cytokines TNF- α , IL-1 β , and

IL-6 increased in the sera of patients with *V. vulnificus* septicemia. Correspondingly, our animal studies using BALB/c female mice also showed elevated levels of TNF- α , IL-1 β , and IL-6 in the serum and the peritoneal fluid after infection by a clinical isolate of *V. vulnificus* 3 h after the experimental initiation of the infection. Furthermore, an inoculum dose-dependent response of serum TNF- α and IL-1 β was observed; this was positively correlated with the fatality rate in the mice. As the bacterial inoculum increases from 1.2×10^5 to 1.8×10^7 CFU, the fatality rate due to Vv05191 infection also increases from 0 to 100% within 9 h. These data support the concept that proinflammatory cytokines play a critical role in the pathogenesis of *V. vulnificus*-induced septicemia.

Our studies showed that in mice with *V. vulnificus* infections, the secretion of proinflammatory cytokines into the serum is dramatically downregulated with all the antimicrobial therapies examined. However, evidence exists to indicate that different classes of antibiotics may differentially modulate the endotoxin release from Gram-negative bacteria and subsequent cytokine response [15,24]. Although minocycline, a tetracycline analog, can stimulate IL-1 β , TNF- α and IL-6 production from LPS-stimulated human monocytes [11,12], tetracycline has been found to be able to reduce LPS induction of TNF- α release in mice [25]. We demonstrated that in mice with experimental *V. vulnificus* infections, minocycline efficiently decreases the circulating levels of proinflammatory cytokines, which is beneficial for the infected mice.

Previous in vitro studies have shown that cefotaxime exerts a positive effect in regulating human monocytes [26], and incubation of Gram-negative organisms with β -lactam antibiotics, especially penicillin-binding protein (PBP)-3-specific agents such as ceftazidime or cefotaxime, leads to filament formation and subsequent release of proinflammatory cytokines and large amounts of endotoxin [24,27,28]. In patients with severe melioidosis, treatment with a PBP-3-specific antibiotic was associated with greater systemic endotoxin release than treatment with a PBP-2-specific antibiotic [29]. However, this cytokine-stimulating property of cefotaxime is not permanent since our studies showed that when compared with mice receiving antimicrobial therapy, the level of serum cytokine was higher in cefotaxime-treated mice only at 3 h post-Vv05191 infection. Cefotaxime inhibited the secretion of cytokines at 6 and 9 h; this is probably responsible for its maximal antibacterial activity after 3 h, which is in agreement

with an earlier study. Evans and Pollack found that the maximal killing of *E. coli* was evident at 4 h, and the endotoxin release reached a plateau as early as 1 h after exposure to a high concentration of ceftazidime (256 µg/mL) [30]. These apparently contradictory data on the in vitro and in vivo effects of minocycline and cefotaxime on cytokine production point to the complexity of the interaction between antimicrobial agents, bacteria, and host immune cells.

Both the antibiotics used in this study — cefotaxime and minocycline — have previously been reported to be immunomodulatory antibiotics [11,15,26]. This leads to the question of whether the lowered levels of cytokine observed in our murine model of Vv05191 infection was due to an immunomodulatory effect or antimicrobial activity. In order to differentiate between the downregulation of cytokines due to the reduced loading of active microorganism and the immunomodulating effect, we conducted an in vitro study using heat-inactivated Vv05191. The results showed the absence of immunomodulatory effects of both cefotaxime and minocycline and allowed us to conclude that the decreased cytokine levels in this experiment was due to the antimicrobial activity via the lowering of the bacterial biomass.

The serum cytokine levels in cefotaxime-minocycline-treated and minocycline-treated groups were not significantly different, both showing the same decreasing trend over time. However, there was a significant difference in the cytokine levels between these groups and the cefotaxime treatment group. As compared with the control (non-treated) mice, only mice treated with the cefotaxime-minocycline combined regimen showed significantly lower cytokine levels in the peritoneal fluid.

Consistent with previous reports, serum and peritoneal fluid cytokine concentrations did not correlate with each other [31,32], with the peritoneal fluid cytokine concentrations being 50- to 100-fold higher than serum concentrations [33]. Prins et al reported that the variation in cytokine levels was due to differences in the amounts of biologically active endotoxin present after each antibiotic treatment [28]. We propose that the different magnitudes of cytokines in the serum and peritoneal fluid observed with the cefotaxime-minocycline and minocycline regimens reflect the different amounts of endotoxin biomass that interacted with the antibiotics. There was a larger bacterial biomass in the peritoneal cavity than in the serum after local intraperitoneal Vv05191 inoculation;

also, bacteria may translocate from damaged intestinal barrier into the peritoneal cavity due to high cytokine levels [34]. Furthermore, the elevated intraperitoneal cytokines may spillover to produce extremely high levels of cytokines in the systemic circulation [35] where the amount of bacteria may be lower.

The elimination of excessive amounts of intraperitoneal cytokines playing a beneficial role is supported by an association with decreased mortality of patients with peritonitis [35]. Holzheimer et al confirmed that local estimates of cytokine levels may better reflect the severity of an initially local process (e.g., peritonitis) [36]. Therefore, the superiority of the minocycline-cefotaxime combined regimen over minocycline or cefotaxime alone in our previous murine experiment [9] may be due to the greater downregulation of intraperitoneal cytokines.

Our previous in vitro and in vivo experimental results show that the use of cefotaxime in combination with minocycline has a better therapeutic efficacy in the treatment of *V. vulnificus* infections than either drug used alone [8,9]. The present study demonstrated that the combined regimen has a higher efficiency in the reduction of proinflammatory cytokines in Vv05191 infection, especially in intra-abdominal infection. The superior effect of the combined regimen in the treatment of *V. vulnificus* infection is, at least in part, attributable to the difference in magnitude of its capacity to modulate the levels of proinflammatory cytokines in the serum and peritoneal fluid. Also, to our knowledge, this is the first study to show a synergy in the downregulation of cytokine concentrations with the combination of a bactericidal and a bacterostatic antibiotic, an effect that is most prominent in large loads of local bacterial infection, such as in peritonitis or necrotizing fasciitis.

In conclusion, the serum and peritoneal fluid levels of proinflammatory cytokines were found to be upregulated at 3 h after intraperitoneal inoculation of Vv05191 in the murine model, and the increase was inoculum dose-dependent and positively correlated to the subsequent fatality rate. The levels of proinflammatory cytokines in the serum and the peritoneal fluid declined dramatically with all the antimicrobial therapies tested, irrespective of the therapeutic regimen. However, the magnitude of the reduction in cytokine concentrations, especially peritoneal fluid cytokines, was highest in mice treated with a combination of cefotaxime and minocycline, which acted synergistically. These data support the superiority of the use of combination therapy when treating invasive *V. vulnificus* infections.

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