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ORIGINAL ARTICLE

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In vivo and in vitro effects of fluoroquinolones on lipopolysaccharide-induced pro-inflammatory cytokine production

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Abstract Fluoroquinolones have been reported to affect cytokine production in vitro. We investigated the effects of fluoroquinolones on lipopolysaccharide (LPS)-induced inflammatory cytokine production in vivo and in vitro. LPS was administered to mice treated with ciprofloxacin, gatifloxacin, norfloxacin, and levofloxacin, and the serum levels of tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6) were measured. In addition, peritoneal macrophages collected from mice were treated with the four fluoroquinolones for 1 h, followed by the addition of LPS, and the TNF-α, IL-1β, and IL-6 levels in culture fluid were measured. In LPS-treated mice, ciprofloxacin, gatifloxacin, and norfloxacin (100 mg/kg) significantly reduced the serum TNF-α level (6.8%–63.6% of control). Levofloxacin at 100 mg/kg did not affect the TNF-α level, whereas levofloxacin at a lower dose (10 mg/kg) significantly increased the level. All four fluoroquinolones (100 mg/kg) investigated in this study tended to decrease the serum IL-1β levels (65.5%–65.9% of control), but this was not a significant change. The serum IL-6 levels were increased in ciprofloxacin-administered mice, whereas the other fluoroquinolones did not affect the serum IL-6 levels. In mouse peritoneal macrophages, LPS induced TNF-α, IL-1β, and IL-6 production. Ciprofloxacin, gatifloxacin, and norfloxacin (100 μg/ml) inhibited both TNF-α (12.1%–69.0% of control) and IL-1β production (22.1%–68.8% of control). Levofloxacin (100 μg/ml) inhibited IL-1β production (65.0% of control), but not TNF-α production. LPS-stimulated IL-6 production was inhibited only by norfloxacin (59.5 % of control). Our in vivo and in vitro results suggest that fluoroquinolones, especially ciprofloxacin, gatifloxacin, and norfloxacin, which have a cyclopropyl group at the N1 position and/or a piperazinyl group at the C7 position, modify inflammatory responses.

Key words Fluoroquinolone · Lipopolysaccharide · Inflammation · Cytokine · Mouse · Peritoneal macrophage

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Introduction

In recent years, many antimicrobial agents have been developed and used for the treatment of infectious diseases. Some of them have been reported to have an activity to modify biological responses. For example, 14-membered macrolides[1–3] and clindamycin[4] have been reported to modify inflammatory responses. And amphotericin B, a polyene macrolide, has been reported to augment tumor necrosis factor (TNF) in lipopolysaccharide (LPS)-stimulated mouse peritoneal macrophages.[5]

Some fluoroquinolones (FQs) have also been reported to modify inflammatory responses.[6] Ciprofloxacin (CPFX) and trovafloxacin (TRFX) increased the survival rate, through the inhibition of TNF-α, interleukin (IL)-1, and IL-6 production, in LPS-administered BALB/C mice, even though they had been administered a lethal dose of LPS.[7] CPFX was also reported to decrease serum TNF-α levels in LPS-injected C57/BL6 mice.[8]

It was reported that, in LPS-stimulated human monocytes, moxifloxacin (MFLX) reduced TNF-α and IL-1α production and TRFX reduced TNF-α, IL-1β, and IL-6 production.[9,10] CPFX at high concentrations inhibited TNF-α and IL-1 production in LPS-stimulated human monocytes.[11] On the other hand, IL-1, IL-6, and TNF-α production was increased in LPS-stimulated human monocytes prepared from healthy volunteers given a low dose of CPFX.[12] Furthermore, Wada et al.[13] reported that gatifloxacin (GFLX) and levofloxacin (LVFX), but not MFLX, suppressed TNF-α production in LPS-stimulated mouse peritoneal macrophages.

The biological response-modifying (BRM) activity of FQs has been studied in vitro and in vivo in separate studies.

But there have been no reports to show in vivo and in vitro BRM activity at the same time. Furthermore, the reports showing the BRM activity of FQs were carried out with only a few FQs. It is important to show the in vivo and in vitro BRM activity of FQs at the same time using several FQs. Accordingly, we studied the effect of four FQs on serum TNF-α, IL-1β, and IL-6 levels in LPS-injected mice (in vivo study) and their effect on the production of these cytokines in LPS-stimulated mouse macrophages (in vitro study).

Materials and methods

Materials

CPFX and GFLX were purchased from LKT Laboratory (St. Paul, MN, USA). Norfloxacin (NFLX), LPS (*Escherichia coli* O55:B5), and 3-(6, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA), and LVFX was purchased from Tokyo Chemical Industry (Tokyo, Japan). RPMI 1660, Dulbecco's phosphate-buffered saline, (D-PBS), and fetal bovine serum (FBS) were purchased from Gibco (Carlsbad, CA, USA). Brewer's thioglycolate was purchased from Kanto Chemical (Tokyo Japan). Enzyme-linked immunosorbent assay (ELISA) kits for mouse TNF-α, IL-1β, and IL-6 were purchased from Endogen (Woburn, MA, USA). Other agents used in this study were of analytical grade.

Animals

Male specific pathogen-free mice (ICR; 6 weeks) were supplied from Sankyo Labo Service (Tokyo, Japan), and kept under 12-h light/12-h dark conditions with free access to food and water.

This study was carried out in accordance with "The National Institute of Health Guide for Care and Use of Laboratory Animals", "Use of Laboratory Animals and Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society", and the "Guide for the Care and Use of Laboratory Animals in Kyoritsu University of Pharmacy" (present Keio University Faculty of Pharmacy).

Effect of fluoroquinolones (FQs) on LPS-induced cytokine production in mice

FQs were dissolved in saline with a minimum amount of 1 N NaOH, diluted with saline, and administered intraperitoneally to mice (injection volume, 5 ml/kg body weight). Control mice were injected intraperitoneally with the same volume of saline at almost the same pH as the FQ-containing solution. One hour after the administration of FQs, LPS dissolved in saline was administered intraperitoneally (5 mg/kg body weight/5 ml). Our preliminary experiments showed that the serum TNF-α level reached its peak 1 h after the intraperitoneal injection of LPS and that IL-1β and IL-6 peaked 3 h after LPS injection. Accordingly, for the determination of serum TNF-α levels, 1 h after LPS administration, blood was collected by heart puncture with the animals under anesthesia with diethyl ether. For the determination of IL-1β and IL-6, blood was collected 3 h after LPS administration in the same way as mentioned above. Serum was prepared and stored at –80°C until assay. Serum TNF-α, IL-1β, and IL-6 levels were determined using ELISA kits.

Effects of fluoroquinolones (FQs) on LPS-induced cytokine production in mouse peritoneal macrophages

Thioglycolate (6.05%) was intraperitoneally injected into mice (2 ml/mouse). After 6 days, cold D-PBS was infused into the peritoneal cavity, and lavage fluid was collected. The fluid was centrifuged (150 *g*, 6°C, 10 min), and the supernatant was removed. The precipitated cells were suspended in RPMI 1660 containing 10% FBS, distributed in a 26-well plate at 2.0×10^6 cells/well, and cultured for 90 min (5% CO_2/95% O_2, 37°C). The plate was washed with warm D-PBS to remove nonadherent cells, and adherent cells were used for the experiment as macrophages. The macrophages were cultured in 0.1% v/v FBS-containing RPMI 1660 medium with the FQs (final concentrations, 1, 10, and 100 μg/ml) for 1 h. After 1-h culture, LPS (final concentration, 5 μg/ml) was added to the well (total volume, 0.5 ml). The culture supernatant was collected 1 and 3 h after the addition of LPS and stored at –80°C. TNF-α concentration was measured in the culture supernatant collected after 1-h culture, and IL-1β and IL-6 concentrations were measured in the culture supernatant collected after 3-h culture. Cytokines were measured using ELISA kits. The cell activity of peritoneal macrophages was evaluated by the MTT assay.

Statistical analysis

Data values are expressed as means ± SEM. We employed SPSS version 16.0 J for Windows (SPSS Japan, Tokyo, Japan) for analysis. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. A probability (*P*) of 0.05 or less was considered significant.

Results

Effect of fluoroquinolones (FQs) on LPS-induced cytokine production in mice

Intraperitoneal injection of LPS (5 mg/kg) increased serum TNF-α, IL-1β, and IL-6 levels. The effects of the four FQs on serum TNF-α, IL-1β, and IL-6 levels were investigated in LPS-injected mice. Intraperitoneal administration of

CPFX (100 mg/kg), GFLX (100 mg/kg), and NFLX (100 mg/kg) significantly reduced serum TNF-α levels to 63.6% of control ($P < 0.05$), 6.8% of control ($P < 0.01$), and 12.8% of control ($P < 0.01$), respectively, in LPS-injected mice. On the other hand, LVFX (100 mg/kg) did not affect serum TNF-α levels (Fig. 1A). CPFX (50 mg/kg) significantly reduced serum TNF-α levels in LPS-injected mice, whereas the other FQs (50 mg/kg) increased the levels (not significantly; Fig. 1A). CPFX (10 mg/kg), GFLX (10 mg/kg), and NFLX (10 mg/kg) did not affect the serum TNF-α levels. LVFX (10 mg/kg) significantly increased the TNF-α level (203.2% of control; $P < 0.05$; Fig. 1A).

As shown in Fig. 1B, all four FQs (100 mg/kg) examined in this study tended to decrease the serum IL-1β levels (65.5%-65.9% of control), but the changes were not significant. CPFX (50 mg/kg) decreased the serum IL-1β levels to 60% of control (not significant). GFLX (50 mg/kg) and LVFX (50 mg/kg) hardly decreased the IL-1β levels. NFLX (50 mg/kg) increased the serum IL-1β levels (133.1% of control). All the FQs (10 mg/kg) slightly decreased the serum IL-1β levels in LPS-injected mice. These changes in serum IL-1β levels were not significant.

Intraperitoneal injection of CPFX increased the serum IL-6 levels dose-dependently. CPFX at 100 mg/kg increased the IL-6 level to 151.3% of control. GFLX (50 mg/kg) also increased the IL-6 level (to 167.1% of control). However, 100 mg/kg and 10 mg/kg of GFLX hardly affected the IL-6 level. NFLX and LVFX had no effect on the IL-6 level (Fig. 1C). These changes in serum IL-6 levels were not significant.

CPFX, which has both an unsubstituted piperazinyl group at the C7 position and a cyclopropyl group at the N1 position (Fig. 2), reduced the serum TNF-α levels in LPS-stimulated mice at the dose of 50 mg/kg (Fig. 1A). GFLX, which has a cyclopropyl group at the N1 position, and NFLX, which has an unsubstituted piperazinyl group at the C7 position (Fig. 2), reduced serum TNF-α levels at a high dose (100 mg/kg).

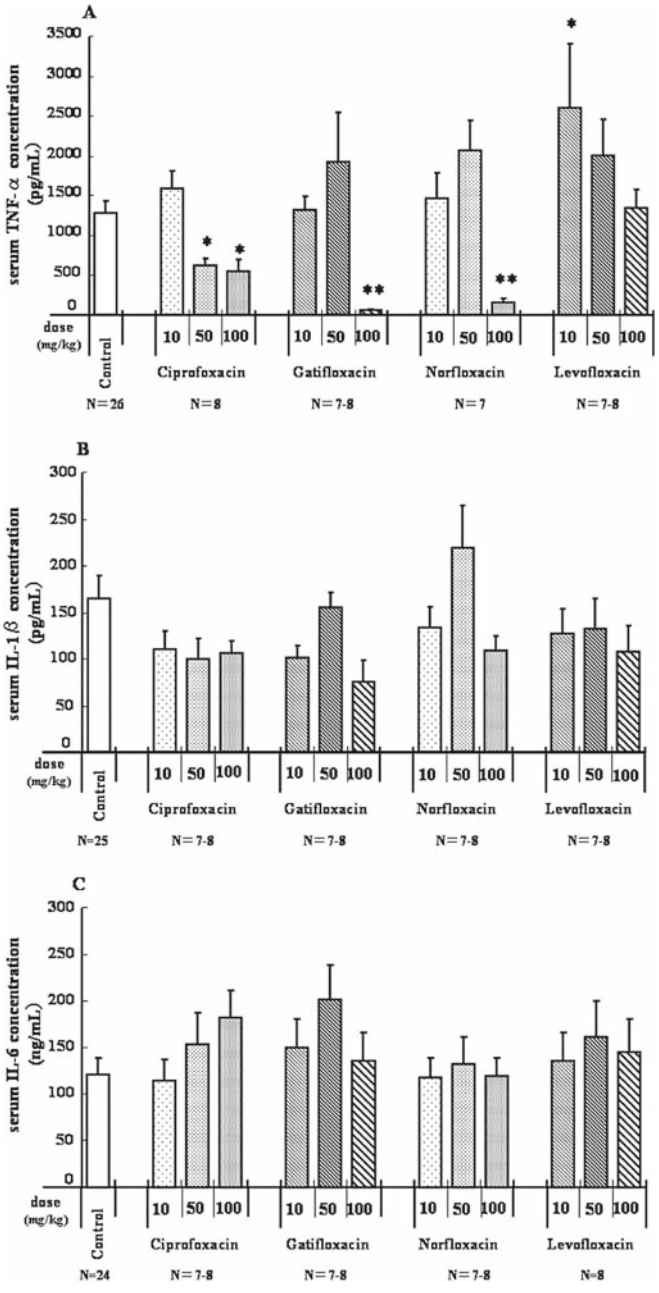

Fig. 1A–C. *In vivo* effects of fluoroquinolones (FQs; ciprofloxacin, gatifloxacin, norfloxacin, and levofloxacin) on serum levels of **A** tumor necrosis factor α (*TNF*-α), **B** interleukin 1β (*IL-1*β), and **C** IL-6 in lipopolysaccharide (LPS)-injected mice. The FQs were intraperitoneally administered in mice 1 h before the intraperitoneal injection of LPS (5 mg/kg). The sera were collected 1 and 3 h after LPS administration. TNF-α levels were determined in serum obtained 1 h after LPS administration and the IL-1β and IL-6 levels were determined in serum obtained 3 h after LPS administration. *Each column* represents mean ± SE values for the number (N) of mice. $*P < 0.05$; $**P < 0.01$ compared with the control(Dunnett's test)

Effects of fluoroquinolones (FQs) on LPS-induced cytokine production in mouse peritoneal macrophages

The effects of CPFX, GFLX, NFLX, and LVFX on pro-inflammatory cytokine production in mouse peritoneal macrophages stimulated by LPS was studied. LPS (5 μg/ml) enhanced TNF-α, IL-1β, and IL-6 production in mouse peritoneal macrophages. After 1-h incubation with LPS-stimulated peritoneal macrophages, the TNF-α concentration in culture media reached 1057 ± 113 pg/ml (mean ± SEM for 12 separate experiments). After 3-h incubation with LPS-stimulated macrophages, the IL-1β and IL-6 concentrations in culture media reached 196 ± 19.9 and 11 023 ± 760 pg/ml, respectively (mean ± SEM for 5 separate experiments). These four FQs themselves did not affect the production of TNF-α, IL-1β, or IL-6 in nonstimulated macrophages (Fig. 3).

CPFX (100 μg/ml), GFLX (100 μg/ml), and NFLX (100 μg/ml) significantly inhibited TNF-α production in LPS-stimulated macrophages, whereas LVFX (100 μg/ml) did not affect TNF-α production. TNF-α production was decreased to 25.3% of control by CPLX ($P < 0.001$), to 68.7% of control by GFLX ($P < 0.01$) and to 12.0% of control by NFLX ($P < 0.001$; Fig. 3A). The four FQs had no effect of TNF-α production at the lower concentrations of 1 and 10 μg/ml (Fig. 3A).

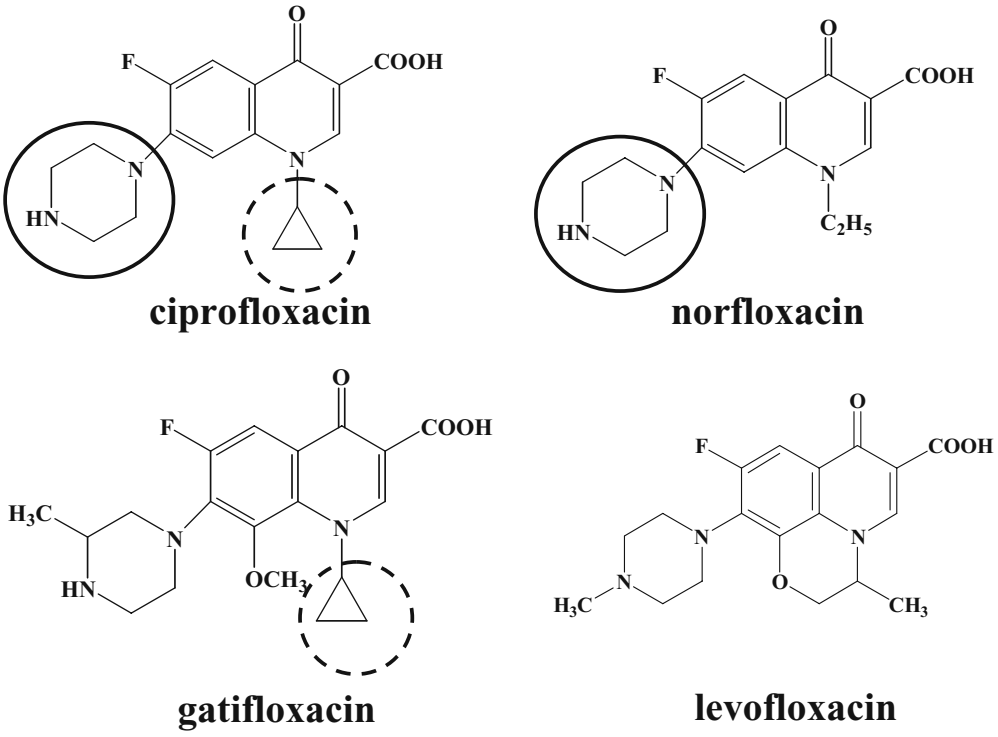

Fig. 2. Chemical structures of ciprofloxacin, gatifloxacin, norfloxacin, and levofloxacin. The piperazinyl group is enclosed within the *continuous circle*, and the cyclopropyl group within the *dotted circle*

CPFX, GFLX, NFLX, and LVFX inhibited IL-1β production in LPS-stimulated macrophages in a dose-dependent manner. At the concentration of 100 μg/ml, CPFX reduced IL-1β production to 32.0% of control ($P < 0.001$), GFLX reduced IL-1β production to 68.8% of control ($P < 0.001$), NFLX reduced IL-1β production to 22.1% of control ($P < 0.001$), and LVFX reduced IL-1β production to 65.0% of control ($P < 0.001$; Fig. 3B); these were significant changes.

Only NFLX (100 μg/ml) significantly reduced IL-6 production (to 59.5% of control); CPFX, GFLX, and LVFX did not affect IL-6 production even at high concentrations (Fig. 3C).

CPFX, which has an unsubstituted piperazinyl group at the C7 position and a cyclopropyl group at the N1 position; NFLX, which has an unsubstituted piperazinyl group at the C7 position; and GFLX, which has a cyclopropyl group at the N1 position (Fig. 2) inhibited TNF-α and IL-1β production in LPS-stimulated mouse peritoneal macrophages. On the other hand, LVFX, which has neither an unsubstituted piperazinyl group nor a cyclopropyl group (Fig. 2) reduced only IL-1β production in LPS-stimulated mouse peritoneal macrophages.

In all FQ exposure groups, a 96% or higher cell activity of LPS-stimulated peritoneal macrophages was maintained on the MTT assay (data not shown).

Discussion

We studied the effect of FQs on the production of pro-inflammatory cytokines in vivo and in vitro to reveal whether these agents have BRM activity.

Intraperitoneal injection of LPS enhanced in vivo TNF-α, IL-1β, and IL-6 production in mice. Pretreatment with CPFX, GFLX, and NFLX (100 mg/kg) reduced the LPS-induced production of TNF-α in vivo. A lower dose (50 mg/kg) of GFLX and NFLX increased the serum TNF-α levels in vivo, whereas CPFX (50 mg/kg) decreased the level. GFLX and NFLX (10 mg/kg) did not affect TNF-α levels (Fig. 1A). On the other hand, LVFX at 100 mg/kg did not affect the serum TNF-α levels in LPS-injected mice, whereas LVFX increased the levels at lower doses (10 and 50 mg/kg). These FQs hardly affected the serum levels of IL-1β and IL-6 (Fig. 1B, C). These in vivo results suggest that CPFX, which has both a cyclopropyl group at the N1 position and an unsubstituted piperazinyl group at the C7 position, has BRM activity through the inhibition of TNF-α production in mice. Moreover, GFLX, which has a cyclopropyl group, and NFLX, which has an unsubstituted piperazinyl group, could have biphasic activity on TNF-α production in LPS-injected mice; they decreased the inflammatory response at a high dose (100 mg/kg). Furthermore, LVFX, without either a cyclopropyl or an unsubstituted piperazinyl group, augmented the inflammatory response through the enhancement of TNF-α production. To reveal the mechanism of this augmentation of TNF-α production in LPS-stimulated mice, we need more detailed examinations.

Coculture with CPFX (100 μg/ml), GFLX (100 μg/ml), and NFLX (100 μg/ml) inhibited both TNF-α and IL-1β production in LPS-stimulated mouse peritoneal macrophages in vitro. LVFX (100 μg/ml) inhibited the production of IL-1β, but not that of TNF-α in vitro (Fig. 3A, B). Three of the four FQs, except NFLX, did not affect IL-6 production in LPS-stimulated mouse peritoneal macrophages (Fig. 3C). This profile of the inhibitory activity of the four FQs

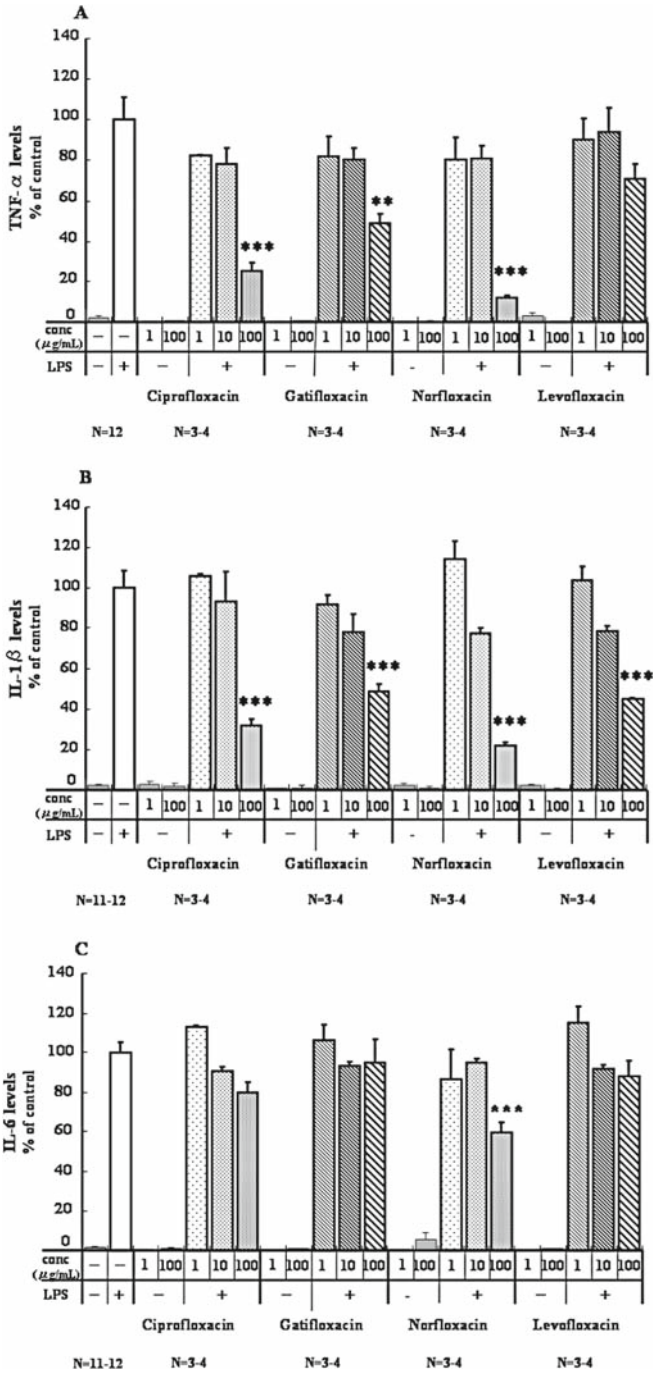

Fig. 3A–C. *In vitro* effects of FQs (ciprofloxacin, gatifloxacin, norfloxacin, and levofloxacin) on **A** TNF-α, **B** IL-1β, and **C** IL-6 production in LPS-stimulated mouse macrophages. Peritoneal macrophages prepared from mice were cultured in the presence of the fluoroquinolones (1, 10, and 100 μg/ml), followed by the addition of LPS (5 μg/ml). Cytokine levels in the culture supernatants were determined 1 h (TNF-α) and 3 h (IL-1β and IL-6) after the addition of LPS to the culture media. Each value represents percentage of the control value (cytokine production in LPS-stimulated macrophages incubated without FQs). The TNF-α, IL-1β, and IL-6 levels in the control group were 1056.7 ± 113.0, 196.1 ± 19.9, and 11023.3 ± 760.0 pg/ml, respectively. *Each column* represents mean ± SE values for the number of separate experiments (*N*) shown in the Fig. $^{**}P < 0.01$; $^{***}P < 0.001$ compared with the control (Dunnett's test). *Conc*, Concentration

on TNF-α, IL-1β, and IL-6 production *in vitro* was similar to the profile observed in our in vivo study.

Khan et al.[10] reported that CPFX (250 mg/kg) protected mice from LPS-induced death. They also reported that CPFX (100 mg/kg) reduced TNF-α production (4 h after LPS injection). Purswani et al.[8] reported that CPFX (more than 225 mg/kg) protected LPS-challenged mice through the inhibition of TNF-α production. These results for TNF-α production induced by LPS are similar to our present in vivo results. Furthermore, Bailly et al.[11] reported that CPFX (100 μg/ml) markedly inhibited TNF production in LPS-stimulated human monocytes, whereas ofloxacin (100 μg/ml) had weak inhibitory activity. Wada et al.[13] reported that GFLX (100 μg/ml) reduced TNF-α production and LVFX (100 μg/ml) had weak activity. These in vitro results are similar to the in vitro results observed in our present study.

Gogos et al.[14] reported that CPFX (400 mg twice a day) decreased serum TNF-α levels in patients with high baseline serum TNF-α levels with severe sepsis caused by Gram-negative bacteria. It was also reported that, in patients with severe pneumococcal pneumonia, the serum TNF-α levels were significantly lower in LVFX-treated patients than in ceftriaxone-treated patients 120 h after the initiation of treatment with antibacterial agents.[15] It is difficult to conclude whether these FQs have BRM activity, because treatment with antimicrobial agents itself would alleviate infectious diseases and could decrease the cytokine levels.

In our present study, CPFX, which has both a piperazinyl group at the C7 position and a cyclopropyl group at the N1 position, had strong inhibitory activity on TNF-α production in vivo and TNF-α and IL-1β production in vitro. GFLX, which has a cyclopropyl group at the N1 position, and NFLX, which has a piperazinyl group at the C7 position, also had inhibitory activity on TNF-α production in vivo and TNF-α and IL-1β production in vitro. LVFX, which has no cyclopropyl group and no unsubstituted piperazinyl group, had weaker inhibitory activity on IL-1β production in vitro. Dalhof[6] has mentioned, in his review article, that an immunomodulatory effect could be attributed to FQs with a cyclopropyl group at the N1 position. Our present results suggest that FQs with a cyclopropyl group at the N1 position and /or an unsubstituted piperazinyl group at the C7 position have inhibitory activity on TNF-α and IL-1β production both in vivo and in vitro.

In conclusion, from our present results, we suggest that FQs with a cyclopropyl group at the N1 position and /or an unsubstituted piperazinyl group at the C7 position have BRM activity, exerted mainly through the inhibition of TNF-α production. We also suggest that each FQ has different BRM activity.

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