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Effect of ciprofloxacin on lethal and sublethal challenge with endotoxin and on early cytokine responses in a murine *in vivo* model

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The influence of ciprofloxacin on immune responses has been suggested by results of *in vitro* and *in vivo* studies. This effect was studied using a murine model that measured mortality and early cytokine responses after challenge with endotoxin. C57/BL6 mice weighing between 18 and 21 g were given a single intraperitoneal dose of lipopolysaccharide (LPS), ranging from 200 to 1000 μg. Mice were pre-treated with an intraperitoneal injection of 5% dextrose in sterile water containing 0.0–6.0 mg of ciprofloxacin 1 h before LPS challenge. Cytokine responses were assessed by measuring concentrations in serum separated from blood obtained by cardiac puncture of anaesthetized mice at 0, 1, 3, 6 and 24 h following LPS administration. Mice were observed for 72 h following administration of LPS and serum cytokines were measured using ELISA. More than 4.5 mg of ciprofloxacin (675–900 mg/m^2 or 225–300 mg/kg) given 1 h before LPS challenge consistently protected mice from a lethal dose of LPS (14/14 versus 0/7, $P < 0.00001$). Ciprofloxacin significantly attenuated the production of tumour necrosis factor-α and interleukin-12 response after LPS challenge. In addition, ciprofloxacin significantly increased serum interleukin-10 concentrations but had little or no effect on interleukin-6 or interleukin-1β serum concentrations. Similar effects were evident with sublethal doses of LPS and were most pronounced at the lowest dose of LPS studied. These observations indicate that ciprofloxacin can prevent endotoxin-mediated death and alter early host cytokine responses. This effect may influence the course of infection in a manner that is independent of the drug's antimicrobial activity.

Keywords: ciprofloxacin, fluoroquinolone, cytokine response, inflammatory response, endotoxin, mortality, innate immunity, immune response, murine, *in vivo*

Introduction

Fluoroquinolones have been widely used to treat a broad array of infectious diseases. As a class of agents they have been shown to be highly effective and safe. However, there is considerable evidence that these drugs can affect normal mammalian cell function in addition to exerting their antibacterial activity.[1–5] Observations in animals and in *in vitro* systems using human and animal tissues have strongly suggested that the influence of some of these agents on immune function could impact on clinical outcomes in patients.[6–10]

Ciprofloxacin is a fluoroquinolone with activity against both Gram-positive and -negative bacteria. It is widely used to treat focal as well as serious disseminated infections. Inhibitory and stimulatory effects of ciprofloxacin on the immune system have been suggested, primarily by studies that have shown that the production of several cytokines by human and murine leucocytes can be affected by this drug.[11] Ciprofloxacin has been shown to enhance interleukin-2 (IL-2) gene induction at clinically achievable concentrations.[12,13] Ciprofloxacin has also been shown to decrease production of tumour necrosis factor-α (TNF-α), IL-1α, lymphotoxin and granulocyte-macrophage colony stimulating factor (GM-CSF) by human lymphocytes.[14] *In vitro* studies have also shown that lower doses of ciprofloxacin enhance production of IL-1, IL-6 and TNF-α by human monocytes.[15] Additional studies

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have suggested that the effects of this agent on cell function are not confined to leucocytes. Recent work has demonstrated that ciprofloxacin can reduce IL-6 and IL-8 secretion by human endothelial cells.[16] Although these observations strongly suggest that ciprofloxacin could influence host defences, the effect of this drug on these responses in patients and on disease associated with these responses has not been demonstrated.

The purpose of the work presented here was to examine ciprofloxacin's influence on inflammatory responses *in vivo*. A murine model was used that involved challenge with lipopolysaccharide (LPS). LPS is a major component of the outer membrane of Gram-negative bacteria. It is a potent stimulus of the inflammatory response and is considered to play a central role in mediating disease caused by Gram-negative bacteria.[17,18] The inflammatory response that occurs after challenge with LPS is associated with elevated serum concentrations of TNF-α, IL-1β and IL-6. Increased concentrations of these proteins have been associated with endothelial damage along with circulatory and metabolic derangement and the systemic inflammatory response syndrome.[19] Using this model, the effect of ciprofloxacin on survival of mice challenged with LPS and on early cytokine responses was studied. In addition to characterizing the influence of ciprofloxacin on TNF-α, IL-1β and IL-6 responses, the influence of the drug on IL-10 and IL-12 responses was also studied. IL-10 and IL-12 are likely to play roles in innate as well as acquired immunity. Therefore, influence on these responses may impact not only the outcome of acute infection, but also the cellular and humoral immunity that develops as hosts recover from an infectious disease.

Materials and methods

This study was approved by the Institutional Review Board for the care and handling of animal subjects. The care and handling of the animals was carried out according to the established animal experimentation guidelines at the Weill Medical College of Cornell University, The New York-Presbyterian Hospital, for ethical animal research.

Reagents

Ciprofloxacin (Cipro I.V.; Bayer Corporation, West Haven, CT, USA) was prepared according to the package insert. Drug concentration was adjusted by diluting with 5% dextrose water (D5W). A stock solution of 5 mg/L LPS (*Escherichia coli* O111:B4; Sigma Chemical Co., St Louis, MO, USA) was prepared by dissolving LPS in sterile, endotoxin-free water. Aliquots of stock were stored at –20°C. Stock solutions of LPS were diluted using phosphate-buffered saline (PBS; Life Technologies, Grand Island, NY, USA) immediately before challenging mice.

Murine model of endotoxin-mediated mortality

C57/BL6 (Taconic, Germantown, NY, USA) mice were challenged in groups of four with LPS (dose range: 200–1000 μg) in a total volume of 1 mL by intraperitoneal (ip) inoculation. Mice were observed for feeding, movement and activity, grooming (smooth and shiny coats versus dull and ruffled coats) and survival for 72 h. Using our established dose of LPS that was uniformly lethal, the effect of ciprofloxacin (3.0, 4.5 and 6.0 mg) on LPS-induced mortality was assessed by giving ip doses of ciprofloxacin in a total volume of 1 mL, 1 h before LPS challenge. Control animals received D5W instead of ciprofloxacin.

Cytokine responses

Cytokine responses were assessed by measuring serum concentrations. Serum was separated from clotted blood obtained by cardiac puncture of anaesthetized mice at 0, 1, 3, 6 and 24 h following administration of ip LPS in groups of five for each dose of LPS used (20, 200 and 1000 μg). Mice were killed after cardiac puncture. Serum was stored at –70°C and concentrations of each cytokine were measured by ELISA according to the manufacturer's instructions (Biosource International, Camarillo, CA, USA).

Statistical analysis

Differences between mean values of normally distributed data were assessed by an unpaired Student's *t*-test. Differences in mortality of groups were assessed by Fisher's exact test.

Results

Effect of ciprofloxacin on endotoxin-mediated mortality

The dose response of LPS on mortality in the murine model is shown in Figure 1. Mice receiving 1000 μg of LPS all died between 18 and 38 h after LPS challenge. Based on these experiments, it was concluded that the LD_{50} of this LPS was between 1000 and 500 μg. At doses as low as 200 μg mice appeared less active after ip challenge. Mice receiving the lowest dose used in our experiments (20 μg) could not be distinguished from mice given PBS alone.

The influence of ciprofloxacin on endotoxin-mediated mortality was assessed by measuring survival of mice challenged with 1000 μg of LPS, a dose of LPS that was uniformly fatal. The dose response of ciprofloxacin on survival of mice receiving 1000 μg of LPS is also shown in Figure 1. Survival to 72 h in mice receiving 3.0 mg of ciprofloxacin was indistinguishable from mice that did not receive ciprofloxacin. Time to death in these two groups was also indistinguishable (data not shown). However, doses of ciprofloxacin ≥4.5 mg

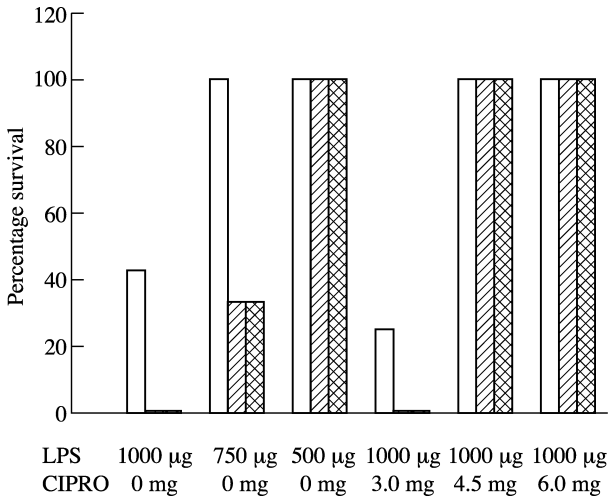

Figure 1. Influence of single dose ciprofloxacin on survival of endotoxin (LPS)-challenged mice. Mice were given 1000, 750 or 500 μg of LPS by intraperitoneal (ip) injection without pre-treatment with ciprofloxacin (CIPRO; first three sets of bars). Ciprofloxacin was administered by ip injection at three doses (3.0, 4.5 and 6.0 mg) 1 h before LPS challenge with 1000 μg (second three sets of bars). White bars, 24 h; striped bars, 48 h; hatched bars, 72 h.

(675 mg/m^2 or 225 mg/kg) were 100% protective. Survival at 48 and 72 h was significantly greater for mice receiving 6.0 mg of ciprofloxacin or 4.5 mg of ciprofloxacin and 1000 μg of LPS compared with mice receiving D5W and 1000 μg of LPS (11/11 versus 0/7, $P < 0.0001$, and 3/3 versus 0/7, $P < 0.01$).

Experiments carried out with lower doses of LPS (750 μg) also demonstrated the protective effects of ciprofloxacin (data not shown). All mice challenged with this dose of LPS and given ≥4.5 mg of ciprofloxacin survived (6/6 versus 2/6, $P = 0.03$). No toxic effects of ciprofloxacin were evident from observations made in mice given ciprofloxacin without LPS challenge. Mice receiving doses as high as 6 mg of ciprofloxacin could not be distinguished from those receiving D5W.

Taken together, these results indicate that single doses of ciprofloxacin ≥4.5 mg can consistently prevent endotoxin-mediated death that occurs within 72 h of LPS challenge.

Effect of ciprofloxacin on cytokine responses induced by a lethal dose of LPS

Early cytokine responses after endotoxin challenge have been well characterized and are known to occur within hours of LPS challenge. Specifically, TNF-α, IL-1β and IL-6 have been shown to be produced early in this response and have been suggested to play critical roles in driving physiological responses that lead to death. To define the influence of ciprofloxacin on cytokine responses *in vivo* that have been associated with fatal outcome, serum concentrations of these cytokines and of IL-10 and IL-12 in mice receiving 1000 μg of LPS were compared with concentrations of these cytokines in mice receiving this same dose of LPS and a single dose of ciprofloxacin (6 mg). As shown in Figure 2, ciprofloxacin-treated mice had marked differences compared with the cytokine responses of mice that were not treated. Mean concentrations of cytokines in ciprofloxacin-treated mice challenged with LPS were significantly different from mice given D5W and LPS at 1 and 3 h for TNF-α ($P < 0.02$), at 1 h for IL-10 ($P < 0.05$) and at 3 and 6 h for IL-12 ($P < 0.004$). Ciprofloxacin consistently and significantly reduced TNF-α and IL-12 responses, increased the IL-10 response and had little or no effect on IL-1β and IL-6 responses in this model (Figure 2).

Cytokine measurements performed at 24 h showed that levels had returned close to baseline undetectable values (data not shown).

Effect of ciprofloxacin on cytokine responses induced by sublethal doses of LPS

To test whether ciprofloxacin could have similar effects on immune responses that were not associated with fatal outcome, cytokine responses of mice receiving sublethal challenges of LPS were also characterized. TNF-α, IL-1β and IL-6 responses in mice receiving 200 and 20 μg of LPS were affected in a similar manner to that observed with lethal doses of LPS (Figure 3). In this model, peak serum TNF-α and AUC of TNF-α responses over the first 6 h after LPS challenge were not affected by LPS dose. In contrast, IL-6 responses were incrementally less with lower doses of LPS (Figure 3), and no dose-related association for IL-1β was observed. As observed with lethal doses of LPS, ciprofloxacin-treated mice receiving sublethal doses had marked decreases in TNF-α responses and no effect on IL-1β responses. Mean concentrations of cytokines in ciprofloxacin-treated mice challenged with either 200 or 20 μg of LPS were significantly different from mice given D5W and LPS at 1 and 3 h for TNF-α ($P < 0.04$). At lower doses of LPS, IL-6 responses tended to be less in ciprofloxacin-treated mice compared with controls. However, these differences were not statistically significant.

Both IL-10 and IL-12 responses have been described to occur early in response to endotoxin challenge. It has been proposed that these responses serve to bridge innate and acquired immune responses.[20] Mice given doses of ciprofloxacin that protected against lethal LPS challenge had a significant elevation of serum IL-10 and a significant decrease of serum IL-12 responses. This same pattern of augmentation of IL-10 and decrease of IL-12 responses was evident in mice challenged with sublethal doses of LPS (Figure 4). Mean concentrations of cytokines in ciprofloxacin-treated mice challenged with 20 μg of LPS were significantly different from mice given D5W and LPS at 1 h for IL-10 ($P < 0.0003$) and at 3 and 6 h for IL-12 ($P < 0.005$). Mean concentrations following challenge with 200 μg of LPS were also different from

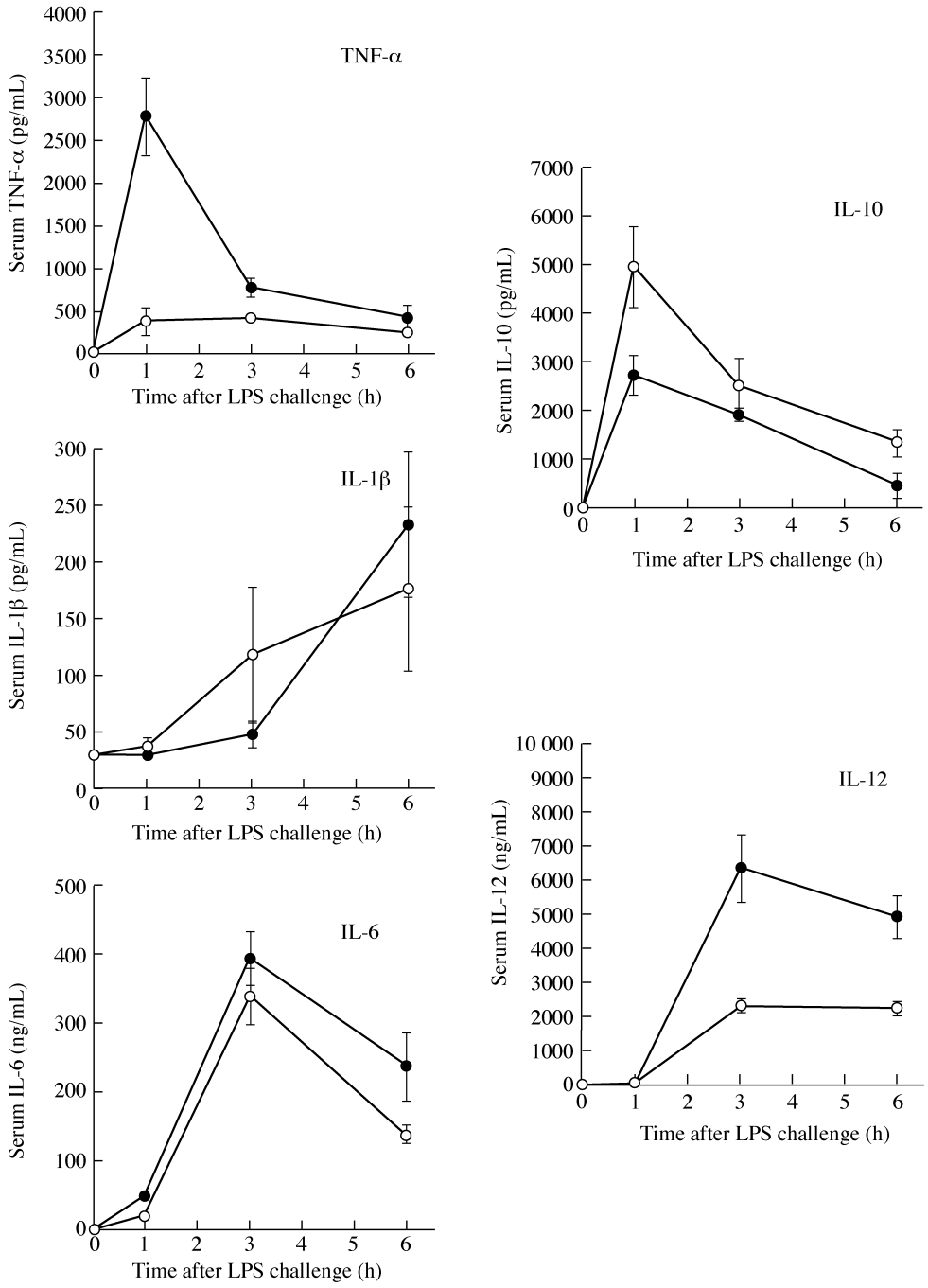

Figure 2. Influence of single dose ciprofloxacin on cytokine responses induced by a lethal dose of LPS. Mice were given either ciprofloxacin (6 mg, white circles) or D5W (black circles) by ip injection 1 h before challenge with 1000 μg of LPS. Serum levels of TNF-α, IL-1β, IL-6, IL-10 and IL-12 were measured at 0, 1, 3 and 6 h following LPS challenge. Circles represent mean values and error bars represent standard errors.

mice given D5W at 3 and 6 h for IL-12 ($P < 0.006$). For both IL-10 and IL-12 the difference between ciprofloxacin-treated and untreated mice was most apparent at the lowest dose of LPS (20 μg). At this dose the mean peak serum IL-10 in ciprofloxacin-treated mice was 5.6 times more than the mean peak serum IL-10 in untreated mice, and the mean peak serum IL-12 in ciprofloxacin-treated animals was 33.8 times less than the mean peak serum concentration of mice receiving LPS alone. Cytokine measurements performed at 24 h showed that levels had returned close to the baseline undetectable values (data not shown).

Discussion

The results presented here clearly demonstrate that ciprofloxacin can prevent mortality associated with endotoxin

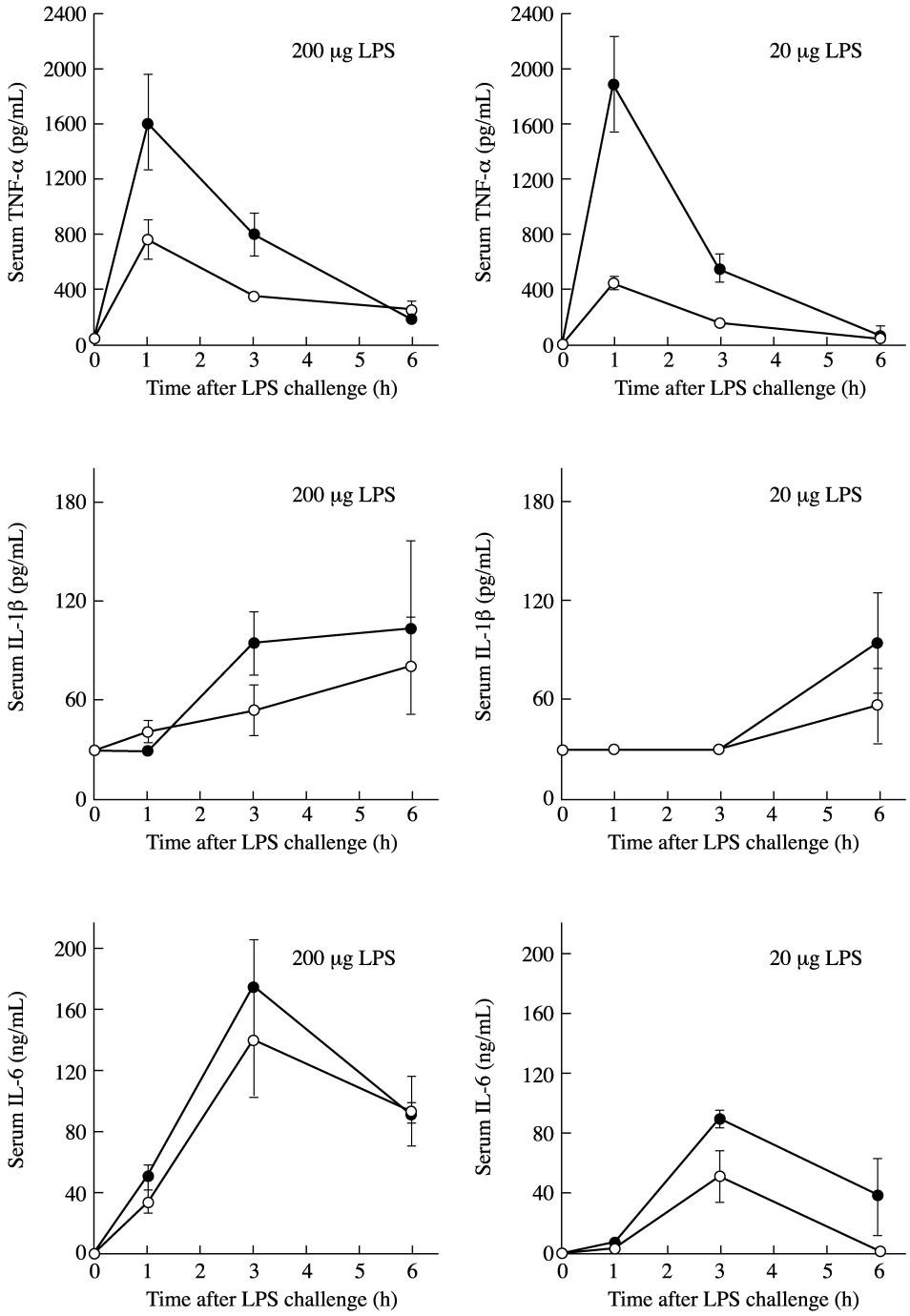

Figure 3. Influence of single dose ciprofloxacin on cytokine responses induced by a sublethal dose of LPS. Mice were given either ciprofloxacin (6 mg, white circles) or D5W (black circles) by ip injection 1 h before LPS challenge with 200 and 20 μg. Serum levels of TNF-α, IL-1β and IL-6 were measured at 0, 1, 3 and 6 h following LPS challenge. Circles represent mean values and error bars represent standard errors.

challenge. This effect was consistent and complete in mice receiving a single 4.5 mg dose or higher of ciprofloxacin, 1 h before challenge with a lethal dose of endotoxin. The doses of ciprofloxacin used in this murine model, although 7.5–10 times the dose per kg, are similar to the doses commonly used in man when expressed as mg/m^2, and are well below the dose of ciprofloxacin that causes any toxicity in mice.[21,22] These data suggest that non-toxic doses of ciprofloxacin prevent a cascade of events that are initiated by endotoxin, and thus that ciprofloxacin is able to produce a potent anti-inflammatory effect *in vivo*.

The basis for improved survival in ciprofloxacin-treated mice is suggested by the influence that ciprofloxacin demonstrated on the pattern of early cytokine responses. In the absence of ciprofloxacin, lethal LPS challenge resulted in an early burst of TNF-α and a modest peak in IL-10 concentra-

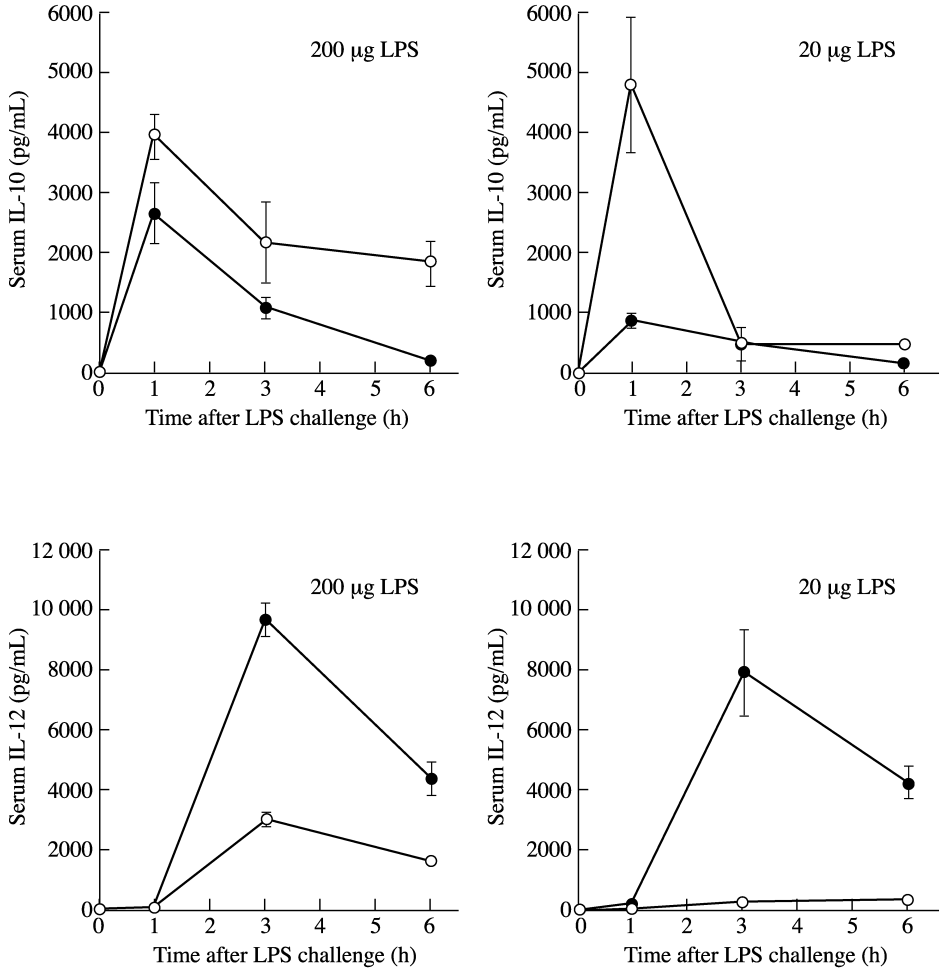

Figure 4. Influence of single dose ciprofloxacin on cytokine responses induced by a sublethal dose of LPS. Mice were given either ciprofloxacin (6 mg, white circles) or D5W (black circles) by ip injection 1 h before LPS challenge with 200 and 20 μg. Serum levels of IL-10 and IL-12 were measured at 0, 1, 3 and 6 h following LPS challenge. Circles represent mean values and error bars represent standard errors.

tion, both occurring at 1 h. These cytokines had largely disappeared by 6 h. Serum IL-1β rose steadily over the 6 h period and serum IL-6 and IL-12 concentrations peaked at 3 h and decreased considerably by 6 h. Pre-treatment with a dose of ciprofloxacin that prevented death in these mice consistently and significantly attenuated the TNF-α burst, decreased IL-12 concentrations and increased the IL-10 response. It has been shown that endotoxin-mediated death can be reversed by manipulation of these cytokine responses in animal models, either by decreasing TNF-α or IL-12, or by increasing IL-10.[23–26] Based on these observations, it is proposed that ciprofloxacin's effect on cytokine responses is a major, if not only, factor causing improved survival in this model.

In addition to influencing mortality and early cytokine responses associated with death, results presented here demonstrate that ciprofloxacin affects cytokine responses in animals challenged with sublethal doses of LPS. This effect may be particularly important when considering the consequences of early inflammatory responses to infection. Cytokines made in the early phases of an infection influence the functional differentiation of T lymphocytes and serve to help initiate the acquired immune response.[27] Both IL-10 and IL-12 have been considered to play an important role in this process. T lymphocytes stimulated in the presence of IL-12 and γ-interferon have been shown to develop preferentially into Th1 cells producing an adaptive response dominated by macrophage activation.[20] IL-10 has been shown to inhibit the generation of Th1 cells.[28,29] Given these observations, the increase in IL-10 and decrease in IL-12 levels could be viewed as perturbing host responses to favour Th2 immunity. The data presented here suggest that ciprofloxacin may affect humoral and cellular immunity in this manner. Accentuation of IL-10 production and diminished IL-12 production were most pronounced in mice receiving the lowest dose of LPS. Whether similar effects occur in hosts surviving infections or responding to vaccines is not known and is currently being examined.

Influencing the inflammatory responses associated with severe bacterial infection and septic shock has evolved as a major challenge in the treatment of patients. Clinical trials have evaluated agents such as monoclonal antibodies to TNF-α,

fusion proteins, IL-1 receptor antagonists, antibodies to platelet-activating factor and corticosteroids.[30,31] Antimicrobial agents that influence immune responses could further affect these adjuvant therapies and may have potential application in treating patients with sepsis. The data presented here suggest that ciprofloxacin, due to its ability to prevent endotoxin-mediated fatality and its anti-inflammatory effect, may be such an agent. The model described here indicates that these effects are pronounced when ciprofloxacin is given before endotoxin challenge and therefore may be more relevant to chemoprophylaxis rather than treatment. Whether giving ciprofloxacin can influence responses that have already been initiated before an antimicrobial is given, or that could be augmented by the use of a potent bactericidal agent that increases LPS-mediated inflammation, has not been determined.

In summary, ciprofloxacin can prevent death mediated by endotoxin and can produce a profound alteration in the acute cytokine responses occurring in the first few hours after challenge with LPS. This observation suggests that ciprofloxacin influences the inflammatory response and that the drug's effect may extend beyond those that are due to its antimicrobial action.

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