**Effect of Moxifloxacin on Production of Proinflammatory Cytokines from Human Peripheral Blood Mononuclear Cells**

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Antibiotics are widely used as bacteriostatic or bactericidal drugs for therapy for bacterial infections. Besides the respective interactions between antibiotics and bacteria and between the immune system and bacteria, antibiotics also directly interact with the immune system. The immunomodulatory effects of antibiotics include alteration of phagocytes, chemotaxis, endotoxin release, cytokine production, hematopoietic recovery after immunosuppression, and tumoricidal effects on certain cancer cells. Moreover, some antibiotic agents can affect the life spans of cells through the induction or inhibition of apoptosis (6, 8–10, 22).

Among numerous antibiotics, quinolones exert various immunomodulatory effects and are widely used in clinical practice, and newer quinolones with enhanced potencies against microorganisms are continuously being developed (7, 14–17).

In this study, we wanted to evaluate the effects of moxifloxacin, a synthetic methoxyfluoroquinolone with a broad antibacterial spectrum used for numerous clinical indications, on the production of some proinflammatory cytokines from peripheral blood mononuclear cells (PBMCs) that were cultured in Mueller-Hinton broth with 5% lysed horse blood (Becton Dickinson, Cockeysville, Md.) at 37°C. The colonies were then harvested by centrifugation, washed twice in phosphate-buffered saline (PBS), killed by incubation at 65°C for 20 min, and stored at −70°C until use.

Isolation and stimulation of PBMCs. Blood was obtained by venipuncture from healthy volunteers. PBMCs were separated on Ficoll-Paque (Sigma) density gradients, washed twice with calcium- and magnesium-free PBS, and resuspended in RPMI 1640 with 10% fetal bovine serum and 25 mM HEPES at a density of 10^6 cells/ml. The cells were exposed to 100 ng of LPS per ml, 1 μg of LTA per ml, or 10^7 CFU of heat-killed bacteria per ml with or without antibiotic treatment. Adequate concentrations of LPS, LTA, and bacteria were finally made with RPMI 1640, and an equal volume of each stimulus was added to same volume of the PBMC preparation.

ELISA for cytokine release. Cells were seeded into 24-well plates at a density of 10^5 cells/well and were incubated for 6 h in the presence of LPS, LTA, and heat-killed S. pneumoniae or E. coli with or without antibiotics. The cell-free supernatant was collected by centrifugation and stored at −70°C. The concentrations of tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) were assayed with enzyme-linked immunosorbent assay (ELISA) kits (BD Pharmingen, San Diego, Calif.) according to the protocol of the manufacturer.

Viability assay. The viabilities of PBMCs treated with moxifloxacin were evaluated by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (R&D Systems, Inc., Minneapolis, Minn.). In brief, aliquots of 10^5 cells/well were distributed in 96-well tissue culture plates (Nunc, Roskilde, Denmark) in 0.1 ml of 10% fetal calf serum–RPMI 1640 medium and incubated at 37°C with or without moxifloxacin for 24 and 48 h. After incubation, MTT solution was added to the culture medium to achieve a final concentration of 1 mg/ml. After 2 h of incubation at 37°C, detergent solution was added to solubilize the colored formazan crystal produced from MTT. The absorbance was read at 550 nm with a spectrophotometer, and the percentage of viable cells was calculated.

Flow cytometry for detection of cytokine-producing PBMCs. PBMCs were incubated in the absence or presence of moxifloxacin (10 μg/ml) and stimulant (1 μg of LTA per ml or 100 ng of LPS per ml) for 6 h at 37°C in 24-well plates. After incubation, 10^6 cells were harvested, washed twice with PBS, and distributed into polystyrene round-bottom tubes for immunolabeling. Two-color staining for the detection of cell surface markers (CD14 and intracellular TNF-α or IL-6) was performed by the protocol of the manufacturer (BD Pharmingen). Flow cytometric analysis was performed on a FACScan instrument (Becton Dickinson, San Jose, Calif.). The percentage of double-stained cells (cells positive for CD-14 and TNF-α and for CD-14 and IL-6) among the stimulated PBMCs was calculated and compared by stimuli.

WESTERN BLOT ANALYSIS OF ICAM-1. PBMCs were exposed to 100 ng of LPS per ml with or without pretreatment with 10 μg of moxifloxacin per ml for 1 h. Nuclear...
TABLE 1. Effect of moxifloxacin on production of TNF-α by PBMCs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TNF-α concn (pg/ml) with the following stimulus:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPS</td>
</tr>
<tr>
<td>Stimulus only</td>
<td>3,066 ± 249</td>
</tr>
<tr>
<td>Stimulus and:</td>
<td></td>
</tr>
<tr>
<td>moxifloxacin (10 μg/ml)</td>
<td>1,072 ± 23a</td>
</tr>
<tr>
<td>levofloxacin (10 μg/ml)</td>
<td>1,472 ± 45c</td>
</tr>
<tr>
<td>ceftriaxone (250 μg/ml)</td>
<td>1,208 ± 237ab</td>
</tr>
</tbody>
</table>

* The concentrations were measured by ELISA. The values are means ± standard errors of the means for quadruplicate measurements.

b P < 0.05 compared to the result for each stimulus only.

TABLE 2. Effect of moxifloxacin on production of IL-6 by PBMCs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-6 concn (pg/ml) with the following stimulus:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LPS</td>
</tr>
<tr>
<td>Stimulus only</td>
<td>24,405 ± 5,148</td>
</tr>
<tr>
<td>Stimulus and:</td>
<td></td>
</tr>
<tr>
<td>moxifloxacin (10 μg/ml)</td>
<td>19,200 ± 4,129f</td>
</tr>
<tr>
<td>levofloxacin (10 μg/ml)</td>
<td>23,381 ± 1,747</td>
</tr>
<tr>
<td>ceftriaxone (250 μg/ml)</td>
<td>29,952 ± 7,043</td>
</tr>
</tbody>
</table>

* The concentrations were measured by ELISA. The values are means ± standard errors of the means for quadruplicate measurements.

b P < 0.05 compared to the result for each stimulus only.

c P < 0.05 compared to the result for ceftriaxone treatment.

d P < 0.05 compared to the result for levofloxacin treatment.
degradation. Although moxifloxacin pretreatment initially (5 min) enhanced the level of IκBα degradation by LPS challenge, IκBα degradation started to be inhibited by moxifloxacin pretreatment 10 min after LPS challenge. After 15 min, the density of IκBα expression was nearly similar to that of control cells (Fig. 3). These findings suggest that moxifloxacin could interrupt NF-κB activation by inhibiting IκBα degradation.

**DISCUSSION**

Quinolones, which exert their bactericidal effect by inhibiting DNA gyrase, are known to interfere with certain immune functions. A high concentration of ciprofloxacin can inhibit peripheral blood lymphocyte growth (3) or can enhance the level of production of cytokines, especially IL-2, gamma interferon, and IL-4 (18–20). The explanation for these phenomena is exposure of cells to DNA-damaging agents, which induces the functions of numerous genes that facilitate the repair of such lesions; but we must remember that most of these results were obtained with concentrations above 20 μg/ml, which cannot be reached in human blood. On the contrary, the newer quinolones, such as grepafloxacin and trovafloxacin, have immunosuppressive effects in vitro within a range of twofold of the peak level achieved in serum (3, 18, 19). The exact mechanisms of the difference between the immunostimulatory and the immunosuppressive effects due to the quinolone concentration are still uncertain. We wanted to evaluate the immunomodulatory effects of moxifloxacin and the pathways involved. In this study, moxifloxacin decreased the levels of production of TNF-α and IL-6 by LPS- and heat-killed bacterium-stimulated PBMCs in a concentration-dependent manner. The inhibitory effect of moxifloxacin on the production of IL-6 was greater than that of ceftriaxone. These results were similar to those reported by Purswani et al. (16), who assessed the inhibitory effect of trovafloxacin on the production of cytokines and who compared the effects of trovafloxacin to those of ceftriaxone and ciprofloxacin. Purswani et al. (16) showed that trovafloxacin and ciprofloxacin clearly had different effects on TNF-α and IL-6 production, but we could not find any consistent differences between the effects of moxifloxacin and levofloxacin. Moxifloxacin showed more inhibitory activity than levofloxacin only when it was added to heat-killed E. coli-stimulated PBMCs. The differences in the immunomodulating effects among the quinolones and the meaning of these differences are still hard to understand.

Although we confirmed these inhibitory effects with lower concentrations of moxifloxacin, we used the highest concentrations of antibiotics to maximize the differences in some experiments, and the moxifloxacin concentrations in tissues and cells commonly exceeded the peak levels achievable in serum (2.5 to
5.0 μg/ml) (11). Moxifloxacin inhibited cytokine production by CD-14-positive cells such as monocytes, a well-known source of the cytokine response to stimuli, such as bacteria (1, 21, 23). This inhibitory effect was not associated with cellular toxicity. The MTT assay did not show any significant difference in the responses of control and moxifloxacin-treated PBMCs at any concentration tested (Fig. 2). This means that moxifloxacin could directly interfere with the production of cytokines from the stimulated PBMCs, as Araujo et al. (1) have reported. They explained that the moxifloxacin-induced inhibition was a result of the direct interaction with LPS, its receptor, and/or its stimulatory pathway. We wanted to explain this by focusing on the NF-κB signaling pathway.

NF-κB is a ubiquitous and important transcription factor for genes that encode proinflammatory cytokines, such as IL-1, IL-6, IL-8, and TNF-α. The prototype of NF-κB is a heterodimer consisting of p50 and p65 bound by members of the IκB family, including IκBα, in the cytoplasm. NF-κB activation requires degradation of the IκB protein. Phosphorylation of IκB by drugs, cytokines, bacterial products, and viruses rapidly leads to IκB degradation and the translocation of NF-κB to the nucleus. Activation of NF-κB results in the binding of specific promoter elements and the expression of mRNAs for proinflammatory cytokine genes (2, 12, 13).

As shown in Fig. 3, at 5 min after LPS challenge, PBMCs pretreated with 10 μg of moxifloxacin per ml showed more degradation of IκBα than PBMCs not treated with moxifloxacin. However, the levels of expression of IκBα by moxifloxacin-pretreated LPS-stimulated PBMCs started to increase and were similar to those of control cells after 15 min. These findings could be the explanation for the decreased levels of TNF-α and IL-6 production by stimulated PBMCs achieved with moxifloxacin treatment. Ichiyama et al. (5) reported that clarithromycin inhibited NF-κB activation in pulmonary epithelial cells, but this inhibition was not linked to the preservation of IκBα degradation. Macrolides and quinolones might inhibit NF-κB activation by different pathways, but we could not hypothesize what this pathway is because the result could be affected by certain characteristics of the stimuli (such as characteristics related to the bacterium itself or to some bacterial component) and the target cells. Recently, Hoffmann et al. (4) have made an interesting suggestion concerning a negative-feedback loop within the NF-κB and IκBα signaling pathway.

Although an assay to evaluate the alteration of the dynamics of IκBα and a direct assay for NF-κB were not performed in this study, we can assume from the results of Western blot analysis that moxifloxacin inhibits the degradation of IκBα and exerts inhibitory effects on the production of proinflammatory cytokines. Further studies on the alteration of NF-κB and IκBα by repeated exposure to quinolones and the effect on the production of anti-inflammatory cytokines and chemokines will be helpful to extend our knowledge about immunomodulating antibiotics.

In conclusion, moxifloxacin has an inhibitory effect on the production of TNF-α and IL-6 from human PBMCs stimulated with bacteria and bacterial components by inhibiting the degradation of IκBα.

REFERENCES