Erythromycin Inhibits Tumor Necrosis Factor Alpha and Interleukin 6 Production Induced by Heat-Killed Streptococcus pneumoniae in Whole Blood

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To determine the effects of penicillin and erythromycin on cytokine production induced by heat-killed Streptococcus pneumoniae (HKSP), we studied the effects of those drugs on cytokine production induced by S. pneumoniae in human whole blood in vitro and ex vivo. In whole blood in vitro, erythromycin, but not penicillin, caused a dose-dependent decrease in HKSP-induced production of tumor necrosis factor alpha (TNF) and interleukin 6 (IL-6), while the production of IL-10, IL-12, and gamma interferon was inhibited only at the highest erythromycin concentration tested (10-2 M). The production of TNF and IL-6 in whole blood obtained from healthy subjects after a 30-min infusion of erythromycin (1,000 mg) was lower after ex vivo stimulation with HKSP than that in blood drawn before the infusion. Inhibition of TNF contributed to erythromycin-induced inhibition of IL-6 synthesis. Inhibition of TNF and IL-6 production by erythromycin may have a negative impact on host defense mechanisms during pneumococcal pneumonia.

Bacterial pneumonia has an estimated incidence in the United States of four million cases per year, one-fifth of which require hospitalization. Streptococcus pneumoniae is the most commonly identified pathogen in community-acquired pneumonia, with a reported incidence of 27 to 46% (4, 23, 25). At present, penicillin is considered the first-choice antibiotic therapy for pneumococcal pneumonia in most parts of the world. Macrolide antibiotics, such as erythromycin, are given in cases of penicillin allergy. In addition, macrolides are used with increasing frequency for the treatment of pneumococcal pneumonia due to the emerging resistance of pneumococci to penicillin (9, 16).

Cytokines are small proteins involved in the orchestration of inflammatory processes. They interact in a network that consists of proinflammatory cytokines (e.g., tumor necrosis factor alpha [TNF], interleukin 6 [IL-6], gamma interferon [IFN-γ], and IL-12) and anti-inflammatory cytokines (e.g., IL-10). In patients with pneumonia, cytokines are produced within the lung at the site of the infection, where they are important for host defense (5, 8). Indeed, endogenous TNF, IL-6, and IL-12 are essential for limitation of bacterial growth in lungs in mouse models of pneumococcal and Klebsiella pneumonia, while IL-10 hampers antimicrobial defenses in such models (11, 12, 33–35).

Macrolide antibiotics have been found to influence the endotoxin-induced production of cytokines (13, 15, 20, 22, 29). However, the clinical relevance of this finding is uncertain, because macrolides are used mainly for the treatment of infections with gram-positive organisms. The effects of macrolides and β-lactam antibiotics on cytokine production induced by gram-positive organisms are unknown.

In the present study we sought to determine the effects of erythromycin and penicillin on cytokine production induced by heat-killed S. pneumoniae (HKSP) in human whole blood in vitro. In addition, we determined the capacity of whole blood obtained before and after an intravenous erythromycin infusion in healthy subjects to produce cytokines upon ex vivo stimulation with HKSP.

MATERIALS AND METHODS

Reagents. Erythromycin and penicillin were purchased from Abbott (Amstelveen, The Netherlands) and Yamanouchi (Leiderdorp, The Netherlands), respectively. Anti-TNF F(ab')2 fragment (MAK 195F) was kindly provided by Knoll, Ludwigshafen, Germany. MAK 195F is derived from a murine TNF-neutralizing monoclonal antibody (MAb), immunoglobulin G3 (IgG3), and neutralizes the biological activity of recombinant and naturally occurring human TNF (19). The concentration of MAK 195F used (10 μg/ml) represented a 1- to 2-log-unit excess neutralizing capacity over TNF concentrations detected after stimulation with pneumococci. Mouse IgG was purchased from F/hKa Chemia, Buchs, Switzerland.

Whole-blood stimulation. HKSP was obtained from a clinical isolate (serotype D9). The bacteria were cultured overnight in 1 liter of Todd-Hewitt broth (20 h) in 5% CO2 at 37°C, harvested by centrifugation, washed twice in pyrogen-free 0.9% NaCl, resuspended in 10 ml of 0.9% NaCl, and heat inactivated for 60 min at 80°C. A 500-μl sample on a blood agar plate did not show growth of bacteria.

Whole-blood stimulation was performed as described previously (7, 30, 31). Briefly, blood was collected aseptically from healthy subjects with a sterile collecting system consisting of a butterfly needle connected to a syringe (Becton Dickinson & Co., Rutherford, N.J.). Anticoagulation was obtained with endotoxin-free heparin (Heparine; Leo Pharmaceutical Products B. V., Weesp, The Netherlands) (final concentration, 10 U/ml of blood). Whole blood, diluted 1:1 in sterile RPMI 1640 (Gibco BRL, Life Technologies Inc., Paisley, Scotland), was stimulated for 4 to 24 h at 37°C with HKSP (amounts equivalent to a final concentration of 105 or 106 CFU/ml) in sterile polypropylene tubes (Becton Dickinson & Co.). For these experiments, polypropylene tubes were profiled with 0.75 ml of RPMI 1640 with or without the appropriate concentrations of HKSP, erythromycin, penicillin, or anti-TNF, after which 0.75 ml of heparinized blood was added. Tubes were then gently mixed and placed in an incubator. After incubation, plasma was prepared by centrifugation and stored at −20°C until assays were performed.

Erythromycin infusion study. In a separate series of experiments, six healthy subjects, aged 32 ± 2 years (mean ± standard error [SE]), received a 30-min intravenous infusion of erythromycin (1,000 mg in 250 ml of 0.9% NaCl). Blood was collected as described above directly before the infusion, immediately after
the infusion, and at 1, 2, and 4 h after infusion was completed. Stimulation of whole blood was performed with HKSP (10^7 CFU/ml) for 16 h at 37°C as described above. All studies were approved by the institutional scientific and ethics committees.

Cell viability. Cell viability was determined by trypan blue exclusion (24) and incorporation of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium bromide (MTT) (21).

For trypan blue exclusion, aliquots of 0.75 ml of blood in 0.75 ml of RPMI 1640 with HKSP (10^7 CFU/ml) and/or erythromycin (10^-5 to 10^-3 M) were incubated for 16 h at 37°C. The supernatant was removed, and the cell pellet was resuspended in 2 ml of phosphate-buffered saline (PBS). The mononuclear cells were then isolated by standard Ficol-Hypaque centrifugation and washed twice in PBS. The tubes containing the cell suspension were spun in polycarbonate tubes (Becton Dickinson & Co.) at 1,000 × g for 10 min. Cells were stained with 0.04% trypan blue (Sigma, St. Louis, Mo.), and 100 viable or nonviable cells from incubations with different concentrations of erythromycin were counted with a standard microscope. MTT is a reagent that is metabolized to a dark blue end product by viable cells. For MTT incorporation, aliquots of blood from four volunteers, with and without HKSP and/or erythromycin, were incubated for 16 h at 37°C as described above, subjected to NH4Cl lysis to clear erythrocyte contamination (1.5 ml of culture plus 1.5 ml of NH4Cl lysis buffer), and centrifuged at 200 × g for 5 min. The pellet was resuspended in 3 ml of cold RPMI 1640, washed a second time, and resuspended in 0.5 ml of RPMI 1640. Duplicate aliquots of this cell suspension (200 μl) were placed in 96-well round-bottom plates, and 20 μl of MTT (5 mg/ml; Sigma) was added. The plates were incubated for 4 h at 37°C in a humidified atmosphere containing 5% CO2. After removal of 150 μl of the supernatant, 100 μl of 0.4% N HCl-isopropanol was added to solubilize the blue crystals and the absorbance was read at 550 nm.

Assays. The following cytokines were tested with specific enzyme-linked immunoassays (ELISAs) according to the instructions of the manufacturers (manufacturers’ names are in parentheses): TNF (Medgenix, Brussels, Belgium), IL-6 (Pharmingen, San Diego, Calif.), IL-10 (Pharmingen), and IFN-γ (Control Laboratory of The Netherlands Red Cross Blood Transfusion Service [CLB], Amsterdam, The Netherlands). Concentrations of IL-12 p40 and IL-12 p70 were determined by sandwich ELISAs. In short, 96-well Immuno Maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with IL-12 p40-specific MAb C11.79 (2 μg/ml) or IL-12 p70-specific MAb 20C2 (1.25 μg/ml). The plates were washed with 0.2 M PBS-0.05% Tween 20, incubated with 2% milk in PBS for 1 h as a blocking step, and washed again. Samples and standards were diluted in high-performance ELISA buffer (CLB). Human recombinant IL-12 was used as the standard. Samples and standards were incubated together with biotinylated anti-human IL-12 p40 MAb C8.6 (final concentration, 0.5 μg/ml) for 1.5 h at room temperature. After five washes, bound IL-12 p40 or IL-12 p70 was detected with peroxidase-conjugated streptavidin (CLB) and ortho-phenylenediamine as the substrate. The color reaction was stopped after 8 h with 1 M H2SO4, and the absorbance was read at 490 and 650 nm. MAb 20C2 and human recombinant IL-12 were kindly provided by Giorgio Trinchieri, The Wistar Institute, Philadelphia, Pa.

Statistical analysis. All values are means ± standard errors of the means. Two-sample comparisons were performed by using the Wilcoxon test for matched samples. A P value of <0.05 was considered to represent a statistically significant difference.

RESULTS

Time course of cytokine induction by HKSP. Incubation of whole blood without HKSP did not result in detectable cytokine production (data not shown). Incubation of whole blood with HKSP was associated with a dose- and time-dependent production of TNF, IL-6, IL-10, IFN-γ, IL-12 p40, and IL-12 p70. TNF was the first cytokine detectable, peaking after 8 h (36.3 + 9.3 ng/ml), while the other cytokines reached peak concentrations at later time points (IL-12 p40 at 12 h [1.9 ± 0.5 ng/ml], IFN-γ at 16 h [13.8 ± 8.0 ng/ml], and IL-10, IL-6, and IL-12 p70 at 24 h [1.2 ± 0.3 ng/ml, 87.1 ± 10.1 ng/ml, and 31 ± 11 pg/ml, respectively]). Time curves for measured cytokines are shown in Fig. 1. Based on these experiments, a 16-h incubation with 10^6 CFU of HKSP/ml was chosen for further experiments.

Erythromycin infusion did not affect leukocyte counts or differentials. Consequently, expression of cytokine levels corrected for the number of mononuclear cells yielded similar results (data not shown).

Effect of erythromycin infusion on ex vivo cytokine production. Inhibition of HKSP-induced cytokine production in vitro occurred at relatively high erythromycin concentrations. To evaluate the clinical relevance of our findings, we next infused six healthy subjects with 1,000 mg of erythromycin (the dose given to patients with severe infections) and determined the capacity of whole blood to produce cytokines after stimulation with HKSP ex vivo. In these experiments, erythromycin infusions influenced only HKSP-induced production of TNF and IL-6 (P < 0.05 [Fig. 3]), while IL-10, IFN-γ, IL-12 p40, and IL-12 p70 secretion was reduced only at the highest erythromycin concentration tested (10^-3 M). The effects of erythromycin on cytokine production were not caused by a negative influence on the viability of leukocytes, as determined by trypan blue and MTT incorporation (data not shown).

Erythromycin-induced inhibition of TNF production contributes to reduced IL-6 levels. Since it has been reported that endogenously produced TNF in part mediates the production of IL-6 induced by endotoxin (27, 36), we investigated whether...
erythromycin-induced inhibition of TNF production was involved in the negative effect of erythromycin on the synthesis of other cytokines in HKSP-stimulated whole blood. To evaluate this possibility, we incubated whole blood with HKSP in the presence or absence of a neutralizing anti-TNF MAb (10 μg/ml). First, we demonstrated that polyclonal mouse IgG (final concentration, 10 μg/ml) did not influence HKSP-induced cytokine production (data not shown). Anti-TNF inhibited HKSP-induced production of IL-6, indicating that TNF is indeed partially responsible for HKSP-induced IL-6 production in whole blood (P < 0.05 [Table 1]). In the presence of anti-TNF, physiological concentrations of erythromycin failed to influence IL-6 concentrations in HKSP-stimulated whole blood (relative to IL-6 levels measured after incubation without erythromycin and in the presence of anti-TNF), suggesting that erythromycin exerts its effect on IL-6 production at least in part through the reduction of TNF concentrations.

DISCUSSION

In patients with unilateral pneumonia, much higher cytokine concentrations have been found in bronchoalveolar lavage fluid obtained from the infected lung than in lavage fluid from the uninvolved lung or in plasma (5, 8). This suggests that during clinical pneumonia cytokines are produced at the site of the infection. Mouse studies have indicated that locally produced cytokines are required for an effective host defense against bacterial pneumonia (11, 12, 33–35). Therefore, we considered it of interest to examine the effects of antimicrobial agents used for the treatment of pneumonia on cytokine production. In this study, we determined the capacities of erythromycin and penicillin to influence cytokine production. S. pneumoniae was used as a stimulus, since both antibiotics are commonly used to treat pneumococcal pneumonia. We specifically chose to use heat-killed bacteria rather than viable pneumococci, to allow us to study only direct effects on cytokine production and rule out indirect influences (i.e., consequences of an antimicrobial effect).

It was found that erythromycin, but not penicillin, inhibited the production of cytokines implicated in the pathogenesis of pneumonia. Importantly, in whole blood in vitro, erythromycin most potently attenuated the production of TNF and IL-6, and significant inhibition began at concentrations of 10^{-5} and 10^{-4}. FIG. 2. Erythromycin, but not penicillin, influences the release of TNF, IL-10, IL-6, IFN-γ, IL-12 p40, and IL-12 p70 in HKSP-stimulated whole blood. Whole blood diluted 1:1 in sterile RPMI 1640 was stimulated for 16 h at 37°C with HKSP (10^7 CFU/ml) and erythromycin (●) or penicillin (○) (both at concentrations of 10^{-5} to 10^{-3} M). Values are expressed relative to production in the absence of penicillin and erythromycin (means ± SEs for six healthy donors). *, P < 0.05 versus value obtained after incubation without erythromycin. Concentrations after stimulation without penicillin (set at 100%) were 34.1 ± 5.2 ng/ml for TNF, 2.8 ± 0.5 ng/ml for IL-10, 75.7 ± 11.3 ng/ml for IL-6, 4.0 ± 1.6 ng/ml for IFN-γ, 2.1 ± 0.4 ng/ml for IL-12 p40, and 43 ± 21 pg/ml for IL-12 p70.

FIG. 3. Erythromycin infusion inhibits the production of TNF and IL-6. Six healthy volunteers each received a 30-min intravenous infusion of 1,000 mg of erythromycin in 250 ml of 0.9% NaCl (hatched bar). Blood was collected directly before and directly after infusion and at 1, 2, and 4 h after the end of infusion. Whole blood diluted 1:1 in sterile RPMI 1640 was stimulated for 16 h at 37°C with HKSP (10^7 CFU/ml). Values are expressed relative to production before infusion of erythromycin (mean ± SEs for six healthy donors). Concentrations after stimulation and before infusion of erythromycin (set at 100%) were 18.4 ± 1.7 ng/ml for TNF and 165.8 ± 24.4 ng/ml for IL-6. *, P < 0.05 versus value obtained before infusion of erythromycin.
that cytokine production induced by gram-positive organisms may, in part, involve mechanisms different from those used by endotoxin (17). Studies on the mechanisms underlying the effect of erythromycin have produced inconsistent data with respect to the predominant mode of action of this drug. Macrolide antibiotics exhibit their antimicrobial activity by interfering with the protein production of microorganisms. They bind reversibly to the 50S ribosomal subunit of sensitive microorganisms, resulting in a dissociation of the tRNA from the ribosomes during translocation to the mRNA (26). In airway epithelial cells, erythromycin increases cyclic AMP (cAMP) levels (28). Elevation of cAMP levels has a marked effect on cytokine production induced by endotoxin, which includes inhibition of TNF and up regulation of IL-6 and IL-10 (3, 32). Hence, a possible increase in cellular cAMP levels by erythromycin would only partly explain our main findings.

Pneumonia is associated with local production of cytokines. We report here that erythromycin, but not penicillin, inhibits TNF and IL-6 production in whole blood stimulated with S. pneumoniae in vitro. This finding could be reproduced in blood obtained from healthy subjects infused with a clinically relevant dose of erythromycin. Inhibition of TNF and IL-6 production by erythromycin may negatively influence specific host defense mechanisms during pneumococcal pneumonia. Together with other reported anti-inflammatory effects of macrolides (1, 28), these data suggest that, during the treatment of pneumonia, the immunomodulatory actions of this group of antibiotics may be a disadvantage with respect to clearance of the infection.

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REFERENCES


