Intravenous infusion of erythromycin inhibits CXC chemokine production, but augments neutrophil degranulation in whole blood stimulated with *Streptococcus pneumoniae*

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\end{itemize}

Macrolides may influence the inflammatory response to an infection by mechanisms that are unrelated to their antimicrobial effect. Indeed, erythromycin and other macrolides inhibit cytokine production and induce degranulation of neutrophils in vitro. CXC chemokines are small chemotactic cytokines that specifically influence neutrophil functions. To determine the effect of a clinically relevant dose of erythromycin on the production of CXC chemokines and neutrophil degranulation, six healthy humans received a 30 min iv infusion of erythromycin (1000 mg). Whole blood obtained before and at various times after the infusion was stimulated ex vivo with heat-killed *Streptococcus pneumoniae*. Ex vivo production of the CXC chemokines interleukin 8 (IL-8) and epithelial cell-derived neutrophil attractant 78 (ENA-78), in whole blood obtained after erythromycin infusion, was lower than that in blood drawn before erythromycin infusion (maximum inhibition post-infusion: 32.9 ± 6.5% and 35.2 ± 12.6% decrease in production, respectively, expressed as percentage change relative to production before infusion of erythromycin, both \(P < 0.05\)). In contrast, infusion of erythromycin was associated with an enhanced capacity of whole blood to release the neutrophil degranulation products bacterial/permeability increasing protein (BPI), human neutrophil elastase (HNE) and human lactoferrin (HLF) upon stimulation with *S. pneumoniae*. Effects of erythromycin were greatest 4 h after infusion was stopped, when BPI, HNE and HLF concentrations were increased by +107.6 ± 33.5%, +134.7 ± 34.8% and +205.9 ± 55.9%, respectively (expressed as percentage change relative to production before infusion of erythromycin) (all \(P < 0.05\)). These results indicate the ability of erythromycin to reduce CXC chemokine production and to enhance neutrophil degranulation in human blood.

**Introduction**

The inflammatory response elicited in the host as a result of bacterial infection plays an essential role in the early defence against the invading organism.\textsuperscript{1–3} Neutrophils are of paramount importance in this initial reaction.\textsuperscript{4} They are attracted to the site of infection by a number of complex processes, including chemotaxis towards a chemotactic gradient produced by locally synthesized chemoattractants. Chemokines, an expanding family of small cytokines, have an important role in the chemotaxis of several leucocyte subsets.\textsuperscript{5–7} The CXC chemokines act specifically on neutrophils and have been implicated as pivotal mediators of host defence against bacterial infection. Indeed, high concentrations of the prototypic CXC chemokine interleukin 8 (IL-8) have been found in bronchoalveolar lavage fluids and

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pleural empyema in patients with pneumonia.8,9 Furthermore, passive inflammation against the CXC chemokine macrophage inflammatory protein 2 (MIP-2) renders mice susceptible to *Klebsiella pneumoniae* pneumonia10 and, conversely, mice over-expressing the mouse IL-8 homologue KC are relatively resistant to this infection.11 Once neutrophils have reached the site of infection, degranulation of activated neutrophils contributes significantly to antimicrobial defence.12

Macrolide antibiotics are used in the treatment of infections caused by many different pathogens. There is evidence that macrolides can affect the host response to infection by mechanisms that are unrelated to their antimicrobial properties. Macrolides influence cytokine production induced by endotoxin12–14 or Gram-positive bacteria.15 One study reported that erythromycin inhibited IL-8 production in *Pseudomonas*-stimulated neutrophils but not in alveolar macrophages.16 The effect of macrolides on chemokine production induced by Gram-positive stimuli is unknown. In addition, macrolides can influence the function of phagocytes in vitro, including the degranulation of neutrophils. In particular, several 14-member ring macrolides enhance neutrophil exocytosis directly.17–20 The effect of macrolides on neutrophil degranulation when administered to humans in vivo is unknown.

In the present study we sought to determine the effect of an intravenous infusion of a clinically relevant dose of erythromycin on the capacity of whole blood to produce CXC chemokines [IL-8 and epithelial cell-derived neutrophil attractant 78 (ENA-78)] upon stimulation ex vivo with heat-killed *Streptococcus pneumoniae* (HKSP). We also determined the effect of erythromycin infusion on neutrophil degranulation ex vivo induced by HKSP, by measuring release of the contents of azurophilic neutrophil granules [bactericidal/permeability-increasing protein (BPI) and human neutrophil elastase (HNE)] and specific granules [human lactoferrin (HLF)].

**Materials and methods**

**Reagents and bacteria**

Erythromycin was purchased from Abbott (Amstelveen, The Netherlands). Heat-killed *S. pneumoniae* (HKSP) obtained from a clinical isolate (serotype D9) were cultured overnight in 1 L Todd–Hewitt broth (Difco, Detroit, MI, USA) in 5% CO₂ at 37°C, harvested by centrifugation, washed twice in pyrogen-free 0.15 M NaCl, resuspended in 10 mL 0.15 M NaCl, and heat-inactivated for 60 min at 80°C. A sample of 0.5 mL on a blood agar plate did not produce any bacterial growth.

**Design and whole blood stimulation**

**Chemokine production.** Chemokine production was studied as described before.15,21–23 Briefly, blood was drawn using a sterile collection system consisting of a butterfly needle connected to a syringe (Becton Dickinson, Rutherford, NJ, USA). Coagulation was prevented using endotoxin-free heparin (Leo Pharmaceutical Products BV, Weesp, The Netherlands) (10 U/mL blood, final concentration). Whole blood, diluted in sterile RPMI-1640 (GibcoBRL Life Technologies, Paisley, UK), was stimulated with HKSP (amounts equivalent to 10⁶ or 10⁷ cfu/mL, final concentration) in sterile polypropylene tubes (Becton Dickinson). For measurements of chemokine production, whole blood was diluted with an equal volume of RPMI. For these experiments, polypropylene tubes were filled with 0.75 mL of RPMI 1640 with the appropriate concentrations of HKSP and erythromycin, after which 0.75 mL of heparinized blood was added. Tube contents were mixed gently and incubated for 16 h at 37°C. Plasma was then prepared by centrifugation and stored at −20°C until assays were performed.

In a first series of *in vitro* experiments, blood was obtained from six healthy donors to determine the capacity of HKSP to produce chemokines. Six healthy subjects, aged 32 ± 2 years (mean ± s.e.m.) received a 30 min iv infusion of erythromycin (1000 mg in 250 mL saline); blood was collected directly before the infusion, immediately after the infusion and 1, 2 and 4 h later. All samples were handled identically.

**Neutrophil degranulation.** Neutrophil degranulation was studied as described before.24–26 Blood samples were handled in the same way as those for chemokine measurements, except that whole blood was diluted 1:5 in sterile RPMI and stimulated with HKSP for 2 h.24–26

**Assays**

Chemokines were measured by specific enzyme-linked immunosorbent assays (ELISAs) according to the instructions of the manufacturers of the kits, namely Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB; Amsterdam, The Netherlands) for IL-8 and R&D systems (Minneapolis, MN, USA) for ENA-78. The lower limits of detection were 1 and 15.6 pg/mL for IL-8 and ENA-78, respectively.

BPI concentrations were measured by ELISA, as described previously, using monoclonal antibody 4E3 (specific for human BPI) as coating antibody, biotinylated polyclonal rabbit anti-human BPI IgG as detecting antibody, and recombinant human BPI as standard.24 The lower limit of detection of the assay was 200 pg/mL. Concentrations of HNE were determined using a sandwich ELISA. IgG was purified from serum obtained from a rabbit hyperimmunized with human elastase (Elastin Products, Pacific, MO, USA), by protein A affinity chromatography. Immuno Maxisorp plates (Nunc, Roskilde, Denmark) were coated overnight at room temperature with this IgG fraction at 1.5 mg/L. The plates were washed with 0.2 M PBS, 0.05% Tween 20, incubated with 2% (v/v)
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Induction of BPI, HNE and HLF release in whole blood by HKSP

A 2 h incubation of whole blood with 10^7 cfu/mL HKSP was associated with a mean 30-fold increase in HNE, a 13-fold increase in BPI and an 18-fold increase in HLF release (all P < 0.05) (Table II). Incubation with HKSP 10^6 cfu/mL led to a mean 19-fold increase in HNE, an eight-fold increase in BPI and a seven-fold increase in HLF release, compared with control levels (all P < 0.05). Based on these data, further studies were performed with HKSP 10^7 cfu/mL.

Effect of erythromycin infusion on CXC chemokine production ex vivo

Six healthy subjects were infused with erythromycin 1000 mg (a dose given to patients with severe infections), and the capacity of whole blood drawn before and at various times after the erythromycin infusion to produce CXC chemokines after stimulation with HKSP ex vivo was determined. In these experiments, erythromycin infusion inhibited HKSP-induced production of IL-8 and ENA-78 (one way ANOVA, P < 0.05; Figure 1). Inhibition was maximal immediately after infusion of erythromycin for ENA-78 (35.2 ± 12.6% decrease relative to production before erythromycin infusion; P < 0.05), while inhibition of IL-8 was maximal 1 h after the end of infusion (32.9 ± 6.5% decrease; P < 0.05). Erythromycin infusion did not affect leucocyte counts or differentials. Consequently, expression of chemokine levels corrected for the number of neutrophils or monocytes yielded similar results (data not shown).

Results

CXC chemokine induction in whole blood by HKSP

Incubation of whole blood without HKSP did not result in detectable chemokine production. Incubation of whole blood with HKSP was associated with concentration- and time-dependent production of IL-8 and ENA-78. IL-8 and ENA-78 were detectable after 4 h incubation, and reached high concentrations after 16 h in stimulations with 10^6 or 10^7 cfu/mL HKSP (P < 0.05) (Table I). For further experiments, 16 h incubations with HKSP 10^7 cfu/mL were conducted.

Table I. Concentration-dependent release of interleukin 8 (IL-8) and epithelial cell-derived neutrophil attractant 78 (ENA-78) in whole blood stimulated in vitro with Streptococcus pneumoniae

<table>
<thead>
<tr>
<th>Conditions</th>
<th>IL-8 (ng/mL)</th>
<th>ENA-78 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No incubation</td>
<td>&lt;0.001</td>
<td>&lt;0.016</td>
</tr>
<tr>
<td>No stimulus</td>
<td>&lt;0.001</td>
<td>&lt;0.016</td>
</tr>
<tr>
<td>HKSP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10^6 cfu/mL b</td>
<td>67.2 ± 15.2c</td>
<td>14.9 ± 5.3c</td>
</tr>
<tr>
<td>10^7 cfu/mL b</td>
<td>93.0 ± 5.8c</td>
<td>30.4 ± 12.6c</td>
</tr>
</tbody>
</table>

*Whole blood diluted 1:2 in sterile RPMI 1640 was immediately put on ice (no incubation) or incubated in the presence or absence of heat-killed S. pneumoniae (HKSP) for 16 h at 37°C.

Data are means ± s.e.m. of six healthy volunteers.

P < 0.05 compared with no stimulus.

Figure 1. Erythromycin infusion inhibits the production of (a) IL-8 and (b) ENA-78. Six healthy volunteers received a 30 min iv infusion of erythromycin 1000 mg in 250 mL of 0.9% NaCl. Blood was collected directly before and after the end of infusion, and after 1, 2 and 4 h. 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 as percentage change relative to production before infusion of erythromycin (mean ± s.e.m.). t = 0 corresponds to the end of the erythromycin infusion. *P < 0.05 compared with the value obtained after stimulation before erythromycin infusion.

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BPI, HNE and HLF release increased after infusion of erythromycin (one-way ANOVA, \( P < 0.05 \)). Non-stimulated neutrophil degranulation did not increase. Effects of erythromycin were maximal 4 h after infusion was discontinued, when BPI concentrations were 107.6 \( \pm \) 33.5% of those before erythromycin infusion, HNE 134.7 \( \pm \) 34.8% and HLF 205.9 \( \pm \) 55.9% (all \( P < 0.05 \)) (Figure 2).

Discussion

Several studies have demonstrated that erythromycin and other erythromycin A-derived macrolides can cause a decline in cytokine production\(^{12-15}\) and stimulate neutrophil exocytosis \it{in vitro}.\(^{17-20}\) We investigated the effect of an intravenous infusion of erythromycin in humans on the ability of peripheral blood leucocytes to produce CXC chemokines and to release proteinases upon stimulation. \it{S. pneumoniae} was used as stimulus, since erythromycin is frequently used to treat pneumococcal infections. We chose to use heat-killed bacteria rather than viable pneumococci, to study only direct effects of erythromycin on CXC chemokine production and degranulation while ruling out indirect influences (i.e. those caused by an antimicrobial effect). We used whole blood, rather than isolated peripheral leucocytes, to study the effect of erythromycin on chemokine production and neutrophil degranulation, to avoid non-specific activation of cells caused by...
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the isolation procedure. Whole blood has been validated previously as a physiological *in vitro* system to investigate cytokine and chemokine production, and to study mechanisms regulating neutrophil degranulation. Our main findings were that *in vivo* exposure of healthy humans to erythromycin causes a decline in the capacity of whole blood to produce the CXC chemokines IL-8 and ENA-78, while augmenting HKSP-induced release of constituents of both azurophilic (BPI, HNE) and specific granules (HLP) of neutrophils.

CXC chemokines are important for neutrophil recruitment to sites of inflammation and infection, including the lung. ENA-78 was originally isolated from A549 cells; these cells were derived from type II pneumocytes, which also secrete IL-8. Treatment with either anti-IL-8 or anti-ENA-78 antibodies attenuated neutrophil infiltration in lungs and the associated tissue injury in models of ischaemia–reperfusion injury. Anti-MIP-2 treatment reduced neutrophil influx and impaired host defence in mice infected with *K. pneumoniae*. In addition, transgenic mice with compartmentalized overexpression of KC in the lung demonstrated increased bacterial clearance and improved survival, in association with enhanced influx of neutrophils to the lung. In view of these findings, erythromycin-inhibited induction of CXC chemokine production can be considered an undesired side effect of this antibiotic.

The mechanism by which macrolides induce neutrophil degranulation has been investigated *in vitro*. It was found that only 14-member ring macrolides, such as erythromycin, roxithromycin and dirithromycin, can induce neutrophil degranulation in a time- and concentration-dependent manner. Studies on structure–activity relationships have established that the effect of macrolides on neutrophil exocytosis is dependent on the L-cladinose at position 3 of the lactose ring. The presence of L-cladinose facilitates stimulation of the phospholipase D-phosphatidate phosphohydrolase transduction pathway, which is essential for neutrophil degranulation. Furthermore, intracellular accumulation of macrolides is probably necessary, since experimental conditions that favour macrolide uptake also favour the degranulating effect. It is unlikely that intragranular accumulation is a prerequisite for this effect, since macrolides that are trapped within neutrophil granules to the greatest extent (e.g. dirithromycin) are not the most effective compounds in eliciting neutrophil exocytosis.

Peak erythromycin concentrations (23.5 ± 0.9 mg/mL) were comparable to those reported elsewhere. Interestingly, peak concentrations were measured at the end of infusion of erythromycin, with a maximum effect on chemokine production at the end of infusion and 1 h after infusion of erythromycin, while degranulation intensified thereafter. We do not have a clear explanation for this discrepancy; however, one can conclude from these findings that the underlying mechanisms for the inhibition of CXC chemokine production and augmentation of neutrophil exocytosis are different.

The stimulatory effect of macrolides on neutrophil degranulation is unique: generally they have anti-inflammatory properties. For example, they attenuate the oxidative burst reaction by neutrophils and can inhibit the production of cytokines by various cell types stimulated with endotoxin *in vitro*. Recently, we found that erythromycin caused a dose-dependent reduction in HKSP-induced tumour necrosis factor-α and interleukin 6 production in human whole blood *in vitro*. Similarly, iv infusion of erythromycin into healthy subjects was associated with reduced production of these cytokines upon stimulation of whole blood *ex vivo*. The inhibition of CXC chemokine production reported here is in line with these data.

In conclusion, we report here that erythromycin inhibits CXC chemokine production and augments neutrophil degranulation in whole blood obtained from healthy subjects infused with a clinically relevant dose of erythromycin, and stimulated with *S. pneumoniae* *ex vivo*. Taken together with earlier findings showing that macrolides can inhibit cytokine production and neutrophil respiratory burst activity, these data exemplify the multiple immunomodulatory effects that erythromycin may have on host defence mechanisms activated soon after the onset of an infection.

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References


