Antagonism between penicillin and erythromycin against
*Streptococcus pneumoniae* in *vitro* and *in vivo*

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The combination of β-lactam antibiotics and macrolides is often recommended for the initial empirical treatment of acute pneumonia in order to obtain activity against the most important pathogens. Theoretically, this combination may be inexpedient, as the bacteriostatic agent may antagonize the effect of the bactericidal agent. In this study, the possible interaction between penicillin and erythromycin was investigated *in vitro* and *in vivo* against four clinical isolates of *Streptococcus pneumoniae* with MICs of penicillin ranging from 0.016 to 0.5 mg/L and of erythromycin from 0.25 to >128 mg/L. *In vitro* time–kill curves were generated with clinically relevant concentrations of penicillin (10 mg/L) and erythromycin (1 mg/L), either individually or in combination. Antagonism between penicillin and erythromycin was observed for the four isolates. *In vivo* interaction was investigated in the mouse peritonitis model. After intraperitoneal inoculation, penicillin and erythromycin were given either individually or in combination. For two of the four isolates, mortality was significantly higher in the groups treated with the combination of penicillin and erythromycin than in the groups treated with penicillin alone [32/36 (86%) vs 3/12 (25%), *P* < 0.05; and 24/36 (67%) vs 3/12 (25%), *P* < 0.05, respectively]. Using the mouse peritonitis model, *in vivo* time–kill curves showed that there was antagonism between erythromycin and penicillin for the examined isolate. The antagonism demonstrated *in vitro* and *in vivo* between penicillin and erythromycin suggests that β-lactam antibiotics and macrolides should not be administered together unless pneumococcal infection is ruled out.

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four clinical isolates of pneumococci with various suscep-
tibilities to penicillin and erythromycin.\textsuperscript{6,13} We studied
interactions \textit{in vivo} in the mouse peritonitis model.\textsuperscript{6}

Materials and methods

\textbf{Bacteria, media and growth conditions for the mouse peritonitis model}

Four clinical isolates of pneumococci were used [isolate numbers 73 (68062), 75 (68098), 86 (68122) and 93 (493/73)].\textsuperscript{13} The MICs of penicillin for these isolates ranged from 0.016 to 0.5 mg/L and those of erythromycin from 0.25 to >128 mg/L (Table).

Bacterial suspensions to be used as inocula for \textit{in vitro} and \textit{in vivo} tests were prepared from fresh overnight cultures on 5\% blood agar plates made from frozen stock cultures. The inoculum for the mouse experiments was prepared immediately before inoculation by suspending the colonies in sterile Mueller–Hinton broth (Statens Serum Institut, Copenhagen, Denmark) and was adjusted to an optical density at 540 nm of 0.5–1.0, giving a concentration of c. 10\(^8\) cfu/mL, as described previously.\textsuperscript{13} For each experiment the size of the inoculum was determined after making 10-fold dilutions in Mueller–Hinton broth, of which 20 \(\mu\)L was plated on two 5\% blood agar plates in spots in duplicate, with subsequent counting of colonies after incubation overnight at 35\(^\circ\)C in air. Mucin (Sigma Chemical Co., St Louis, MO, USA), an enzyme extract of porcine stomach, was used as an adjuvant for inoculation of the mice and was prepared as a stock solution 10\% (w/v) in saline.\textsuperscript{13} Immediately before inoculation, the mucin solutions were diluted 1:1 with pneumococcal suspensions, giving a final mucin concentration of 5\% (w/v).

The drugs used were penicillin G (Leo Pharmaceutical Co., Ballerup, Denmark) and erythromycin (Sigma Chemical Co.). Penicillin G was diluted in phosphate-buffered saline pH 6.5 ± 0.1, and erythromycin was diluted in 9 mL sterile water and 1 mL 96\% alcohol; the pH of the erythro-
mycin solution was adjusted to between 6.7 and 7.3.

\textbf{MICs and MBCs}

MICs were determined by the broth macrodilution method in glass tubes. All tests were done in duplicate and results were read after 20 h of incubation at 35\(^\circ\)C. The broth macrodilution method in glass tubes was performed with Mueller–Hinton broth (Statens Serum Institut) to which 5\% sheep blood was added; an inoculum of \(10^6\) cfu/mL was used. Penicillin G was diluted in two-fold steps in Mueller–Hinton broth to give concentrations of 0.004–64 mg/L. The lowest concentration of antibiotic at which there was no visible growth was taken as the MIC. We used \textit{S. pneumoniae} ATCC 49619 as a control strain for the MIC tests.

The MBC was determined by subculture of tubes with no visible growth after MIC determination. From each tube, 100 \(\mu\)L was cultured on agar plates containing peni-
cillinase (Leo Pharmaceutical Co.) 1000 IU/plate and colonies were counted after 18–24 h incubation at 35\(^\circ\)C. The MBC was defined as the lowest concentration of peni-
cillin that reduced the inoculum by \(\geq 99.9\%\). All assays were performed in duplicate.

\textbf{Time–kill curves}

To study possible interactions between penicillin and erythromycin, time–kill experiments were performed with clinically relevant penicillin and erythromycin concentra-
tions of 10 and 1 mg/L, respectively. Before time–kill experiments, isolate 86 (erythromycin resistant) was grown on 5\% blood agar plates containing erythromycin 4 mg/L (induction of erythromycin resistance) and on plates with-
out erythromycin (no induction of erythromycin resist-
ance). Pneumococci (\(10^6\) cfu/mL) were incubated in 20 mL of Mueller–Hinton broth at 35\(^\circ\)C with shaking. To ensure exponential growth of the bacteria, antibiotics were first added after 1 h of incubation. Samples were taken before the addition of antibiotics and 1, 2, 3 and 5 h later. Time–kill curves were not extended further, since autolysis started to occur after 5–6 h for all isolates. The numbers of cfu/mL were determined after making appropriate dilutions, and 100 \(\mu\)L was spread on to 5\% blood agar plates. For undiluted samples, agar plates containing penicillinase (as

<table>
<thead>
<tr>
<th>Isolate number</th>
<th>MIC (mg/L)</th>
<th>MBC (mg/L)</th>
<th>ED\textsubscript{50} (mg/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>penicillin</td>
<td>erythromycin</td>
<td>penicillin</td>
</tr>
<tr>
<td>73</td>
<td>0.016</td>
<td>0.25</td>
<td>0.016</td>
</tr>
<tr>
<td>75</td>
<td>0.5</td>
<td>0.25</td>
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<tr>
<td>86</td>
<td>0.125</td>
<td>&gt;128</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>without induction</td>
<td>&gt;128</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>with induction</td>
<td>&gt;128</td>
<td>0.125</td>
</tr>
<tr>
<td>93</td>
<td>0.016</td>
<td>0.25</td>
<td>0.032</td>
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</table>

Table. Characteristics of the isolates used

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above) were used. Colonies were counted after 20 h of incubation at 35°C. All time–kill experiments were performed in duplicate. The variation in colony counts at the same time points for repeated experiments was <0.5 log₁₀ per mL. Antagonism was defined as a significantly decreased killing effect (i.e. >0.5 log₁₀ cfu/mL from 1 to 5 h after adding drugs) of the combination of penicillin and erythromycin as compared with penicillin alone.¹³

In the time–kill experiments, we measured possible pH changes in the flasks with pH test strips (pH range 4.5–10.0; graduated in 0.5 pH units; Sigma Chemical Co.).

Animal experiments (mouse peritonitis model)

All animal experiments were approved by the Danish national ethical committee. Outbred female ssc CF-1 mice (Statens Serum Institut) aged 8–12 weeks, weight 28–30 g were used, in groups of five to 36 mice. Overall, we used 88 mice for isolate 73, 89 mice for isolate 75, 88 mice for isolate 86 (without induction), 94 mice for isolate 86 (induced with erythromycin as for time–kill studies) and 89 mice for isolate 93. The mice were kept in cages with five to seven mice per cage; they were allowed free access to food and water. Pneumococcal suspension (0.5 mL) was inoculated intraperitoneally via a 25-gauge syringe. The inoculum contained 10⁶ cfu/mL with 5% (w/v) mucin in Mueller–Hinton broth. Using such inocula, there is c. 100% mortality in untreated mice, which succumb 36–48 h after inoculation. Antibiotics were administered subcutaneously in the neck region in a volume of 0.25 mL per dose.¹⁵ The following schedule was used: erythromycin was given 90 min after bacterial inoculation and in the combination group erythromycin was given 90 min after penicillin 60 min later. Penicillin was given alone 150 min after bacterial inoculation. Control mice were given sterile saline 90 min after bacterial inoculation.

Doses of penicillin and erythromycin were chosen according to the Hill equation sigmoid dose–effect curves, in such a way that penicillin alone would be expected to result in c. 95% survival, while erythromycin alone was expected to prevent mortality in 10% of the mice. Mice infected with isolate 73 were treated with penicillin 10 mg/mouse and/or erythromycin 100 μg/mouse. Mice infected with isolate 75 were treated with penicillin 2 mg/mouse and/or erythromycin 100 μg/mouse. Mice infected with isolate 86 (not induced) were treated with penicillin 150 μg/mouse and/or erythromycin 100 μg/mouse. Mice infected with isolate 86 (grown in the presence of sub-inhibitory concentrations of erythromycin before inoculation) were treated with penicillin 150 μg/mouse and/or erythromycin 100 μg/mouse. Mice infected with isolate 93 were treated with penicillin 400 μg/mouse and/or erythromycin 100 μg/mouse.

Blood samples were obtained through orbital cuts after anaesthetizing the mice with CO₂. Mice were killed and peritoneal washes were then performed by injecting 2 mL of sterile saline intraperitoneally, massaging the abdomen and opening the peritoneum to collect the fluid.¹⁵ Blood and peritoneal fluid samples were immediately diluted, and 0.1 mL was plated on to 5% blood agar plates.

In vivo time–kill curves were constructed for one of the pneumococcus isolates (number 93), which showed significant antagonism in vivo. Pneumococci in the peritoneum and blood were obtained after inoculating 36 mice intraperitoneally with 5.0 × 10⁶ cfu/mL of the bacterial suspension. Ten minutes, 80 min, 140 min, 3 h and 5 h after challenge, groups of three control mice (inoculated with sterile saline) were killed. At 140 min, 3 h and 5 h after challenge, groups of three mice treated with erythromycin 90 min after challenge were killed. At 3 h and 5 h after challenge, groups of three mice treated either with the combination of penicillin and erythromycin or penicillin alone 150 min after intraperitoneal infection were killed. Blood and peritoneal washings were sampled for quantitative culture of pneumococci.

Determination of ED₅₀ of penicillin for individual pneumococci

The ED₅₀, the single dose giving protection to 50% of the mice, for each pneumococcus isolate was determined by treating groups of five mice with doubling doses of the antibiotics; survival of the mice was observed for 7 days. A group of five mice treated with 0.9% NaCl was included in each experiment as a control for the lethality of the infection. For each isolate the ED₅₀ was calculated by the method of Reed & Muench¹⁶ and from the Hill equation (GraphPad Prism; GraphPad Software, Inc., San Diego, CA, USA). We did not perform any experiments in order to calculate the erythromycin ED₅₀ since this has been done previously for strains with similar MICs in our laboratory.¹⁷ We chose penicillin doses that would be expected to result in an estimated 95% survival of the mice and erythromycin doses that would be expected to result in an estimated 10% survival of the mice. For combination therapy, the antibiotics were given in separate injections.

Statistics

Fisher’s exact test for categorical data was used, with a two-sided 5% level of significance.

Results

The MICs of penicillin and erythromycin and the ED₅₀ of penicillin for the four pneumococcal strains are shown in the Table. Lethal infection was achieved with three of four isolates. However, the ED₅₀ of penicillin for isolate 86 had to be estimated since it was only possible to achieve 90% mortality in the ED₅₀ determination experiments.
The results of the time–kill experiments are shown in Figure 1. By this method, all isolates showed in vitro antagonism for the combination of penicillin and erythromycin (Figure 1a–c and e) because erythromycin almost completely inhibited the bactericidal effect of penicillin. However, when erythromycin resistance was induced by growing the erythromycin-resistant isolate (number 86) on blood agar plates containing erythromycin 4 mg/L before the time–kill study, erythromycin antagonism was neutralized (Figure 1d).

For all isolates, the in vitro killing effect of penicillin and penicillin plus erythromycin increased with time. The decrease in cfu seen in the control flasks in some experiments was due to autolysis, a common observation with pneumococci. In all experiments, erythromycin alone showed a smaller killing effect than penicillin and the combination. No killing effect was observed during the 5 h of incubation in the erythromycin-resistant strain, whether incubated with erythromycin or not. No changes in pH were observed in the flasks containing one or both antibiotics: the pH was 7.5 after 0, 1, 2, 3, 4 and 5 h of incubation.

In the mouse peritonitis model, we found that the combined treatment of penicillin and erythromycin resulted in antagonism in mice challenged with isolates 75 and 93 (Figure 2b and e). There was significantly higher mortality in the mice treated with erythromycin 60 min before penicillin than in mice treated with penicillin alone (isolate 75: mortality 32/36 and 3/12, respectively, \( P < 0.05 \); isolate 93: mortality 24/36 and 3/12, respectively, \( P < 0.05 \)) (Figure 2). In the remaining two isolates, mortality in mice given both antibiotics was similar to that in mice given penicillin alone (Figure 2a and d). All control mice died when infected intraperitoneally with pneumococci, except mice infected with isolate 86, for which the mortality was 80–90%. Even when the bacterial inoculum of isolate 86 was increased to \( 10^8 \) cfu/mL, there was no 100% mortality.

The lowest mortality rates were found in those groups of mice treated with penicillin alone or erythromycin plus penicillin in combination after bacterial challenge. The highest mortality was observed among control mice or mice treated with erythromycin alone 90 min after pneumococcal infection (Figure 2). For isolate 86 we found that the combined treatment provided significantly better protection against pneumococcal infection than treatment with penicillin alone after 150 min (32/35 mice in the former group survived compared with 6/12 in the latter; \( P = 0.009 \)) or erythromycin alone (32/35 and 16/36 survival, respectively; \( P = 0.005 \)) (Figure 2c). When erythromycin resistance was induced before inoculation with isolate 86, the combined treatment provided significantly better protection than erythromycin alone (31/36 mice in the former group survived compared with 5/36 in the latter; \( P = 0.005 \)).

The in vivo time–kill curves for isolate 93 showed a 1–2 log increase in growth of bacteria in blood 80 min after challenge compared with 10 min after challenge in the control mice (Figure 3), whereas the number of pneumococci in peritoneal washes at 80 min was the same as that 10 min after inoculation. The number of pneumococci cultured from blood and peritoneal washes at different time points in the different treatment groups were almost parallel, with 1–2 log higher cfu/mL in peritoneal washes than in blood. Killing of bacteria was most efficient in the penicillin-treated group, with a decrease in bacterial growth of 3 logs and 4 logs in blood and peritoneal washes, respectively. The combination therapy showed a similar effect on bacterial growth as when erythromycin was administered alone (antagonism).

Discussion

The interaction between erythromycin and penicillin against pneumococci was studied with four isolates with different susceptibilities to penicillin or erythromycin. The in vitro time–kill curves for the erythromycin-sensitive isolate (Figure 1a, b and e) showed clear antagonism between the two drugs, i.e. the presence of erythromycin completely inhibited the bactericidal activity of penicillin and the resulting curve was similar to the slow inhibitory activity of erythromycin alone. The inhibition of the effect of penicillin (which is acidic) could have been due to increases in pH caused by erythromycin (which is alkaline). However, pH measurements in flasks with the drug combination showed no changes in pH. The antagonistic effect was seen also in the uninduced erythromycin-resistant pneumococcal isolate (86), while induction of the expression of the erythromycin resistance gene prevented the inhibitory effect of erythromycin and its antagonistic activity on penicillin. These results clearly indicate that it is the inhibitory activity of the macrolide on the growth or cell division of the bacteria that is the main reason for antagonism against penicillin, which can only act on bacteria that are in the growth phase and producing a cell wall. This bacteriostatic activity of erythromycin and its inhibitory effect on penicillin were clearly apparent in the in vivo experiments.

In order to evaluate the possible interaction between the two drugs in vivo, we chose the mouse peritonitis model, which has previously been useful in demonstrating interaction between antibiotics, and which also enables one to study drug action on bacteria by performing in vivo time–kill experiments on organisms in the peritoneum and/or blood. Doses of penicillin and erythromycin were chosen according to the Hill equation sigmoid dose–effect curves, in such a way that penicillin alone would be expected to result in c. 95% survival, while erythromycin alone would only be able to prevent mortality in 10% of the mice. With these doses, an effect of the erythromycin dose with no influence of a subsequent penicillin dose would result in a mortality determined solely by the activity of the macrolide. The antagonistic activity of the two drugs was
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Figure 1. *In vitro* time–kill curves for *S. pneumoniae* isolates 73 (a), 75 (b), 86 (not induced) (c), 86 (induced with erythromycin) (d) and 93 (e). Organisms were grown in Mueller–Hinton broth supplemented with penicillin 10 mg/L alone (---), erythromycin 1 mg/L alone (■), or penicillin 10 mg/L and erythromycin 1 mg/L (▲), or without antibiotics (——). The lower limit of detection was 100 cfu/mL (—).
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Figure 2. Survival of mice infected intraperitoneally with *S. pneumoniae* followed by injection of penicillin 150 min after inoculation (—); a combination of erythromycin 90 min and penicillin 150 min after inoculation (▲); erythromycin 90 min after inoculation (■); or sterile saline (—). The total numbers of mice treated with penicillin alone, penicillin plus erythromycin, erythromycin alone, or sterile saline were, respectively: (a) (isolate 73) 12, 35, 36 and 5; (b) (isolate 75) 12, 36, 36 and 5; (c) (isolate 86, not induced) 12, 35, 36 and 5; (d) (isolate 86, induced with erythromycin) 12, 36, 36 and 10; (e) (isolate 93) 12, 36, 36 and 5.

subsequently confirmed, by time–kill curves *in vivo*, to be due to the same growth-inhibitory activity of erythromycin as demonstrated *in vitro*. The importance of induction of erythromycin resistance in the erythromycin-resistant isolate (number 86) was also demonstrated *in vivo*: induction with erythromycin significantly decreased the survival in spite of erythromycin treatment as compared with survival following erythromycin treatment of uninduced pneumococci.

With one erythromycin-susceptible isolate (number 73), it was not possible to show antagonism *in vivo* with the method used. There may be several explanations for this. The possibility that not all pneumococci respond in a similar way to the two drugs used seems unlikely, especially when the activity was equally demonstrable *in vitro*. It is more likely that the situation *in vivo* is different for the different strains, e.g. the virulence of the pneumococci depends on a number of factors which are difficult to standardize, such as the type and size of the capsule, other virulence factors connected with the membrane or toxins, or the
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growth behaviour in vivo, which is very variable for pneumococci. It is likely that we could have demonstrated antagonism in vivo with isolate 73 if we had 'titrated' the inoculum and the timing of the two drugs in relation to each other, but this would require an excessive number of animals. The clear-cut demonstration of the interaction between the two drugs against at least two pneumococcal strains both in vitro and in vivo, which was demonstrated by the increase in mortality as well as by the changes in in vivo time–kill curves, is ample evidence for an effect, and should warn clinicians against the use of these two drugs together to treat pneumococcal infections.

The clinical significance of antagonism between a bactericidal drug such as penicillin or ampicillin and a bacteriostatic protein synthesis inhibitor, e.g. tetracycline, erythromycin or chloramphenicol, has been demonstrated in several studies. Antagonism has been confirmed in vivo in an experimental study with penicillin and chloramphenicol against pneumococcal meningitis in dogs. In spite of this early experience with such drug combinations, the combination of penicillin and erythromycin is recommended even in standard textbooks as empirical treatment in pneumonia of unknown aetiology. The drug combination is particularly chosen to encompass pneumococci and Legionella spp., which are considered to be important and deadly aetiological agents in pneumonia. It is difficult to know whether the results of the present study are directly applicable to the clinical situation. In the clinical situation, sub-optimal doses of erythromycin would not usually be used, but with inhibited action of penicillin one may have to rely upon the bacteriostatic effect of erythromycin, if the pneumococcus is erythromycin susceptible. It is not known if the inducible effect of erythromycin demonstrated in the present study also takes place in the pneumonic focus in humans.

In conclusion, the present study has demonstrated antagonism between penicillin and erythromycin against three erythromycin-sensitive isolates of pneumococci in vitro. This antagonism also occurred with two of the isolates in vivo in a simple experimental model with mice. When erythromycin resistance was not induced, antagonism was also demonstrated in vitro for the erythromycin-resistant pneumococcal isolate, but induction with erythromycin before inoculation obliterated the antagonistic effect.

Acknowledgements

The expert technical assistance of Anja Borum and Jytte Mark Andersen is greatly appreciated. Preliminary data presented in final form in this manuscript were presented at the Thirty-Seventh Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Ontario, Canada, 28 September–1 October 1, 1997 (poster A-29).

References


Figure 3. In vivo time–kill curves in (a) blood and (b) peritoneal fluid for S. pneumoniae isolate 93. Penicillin 150 min after inoculation (—); a combination of erythromycin 90 min after inoculation and penicillin 150 min after inoculation (▲); erythromycin 90 min after inoculation (■); or sterile saline control (——). The lower detection limit was 100 cfu/mL (——).
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Received 21 February 2000; returned 4 June 2000; revised 13 July 2000; accepted 19 August 2000

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