Effect of opsonophagocytosis mediated by specific antibodies on the co-amoxiclav serum bactericidal activity against *Streptococcus pneumoniae* after administration of a single oral dose of pharmacokinetically enhanced 2000/125 mg co-amoxiclav to healthy volunteers

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**Objectives:** To measure the effect of opsonophagocytosis mediated by complement activated by specific antibodies on the co-amoxiclav serum bactericidal activity against *Streptococcus pneumoniae* strains with reduced susceptibility to *β*-lactams (amoxicillin MICs of 2, 4, 8 and 16 mg/L).

**Methods:** An open Phase I study measuring *ex vivo* bactericidal activity after anti-pneumococcal vaccination and an oral dose of 2000/125 mg sustained-release co-amoxiclav was carried out. The *ex vivo* bactericidal activity was investigated through killing curves over 3 h [assuring polymorphonuclear neutrophil (PMN) viability] with serum samples collected 1.5 h and 5 h after dosing. Global killing was measured as the area under the killing curve (AUKC; log cfu × h/mL). The AUKC of the control growth curve of *S. pneumoniae* in Hanks’ balanced salt solution (AUKC₀) was used as control. The effect of the presence of complement and/or PMN was evaluated by the difference in the AUKC and the different AUKCs obtained in the presence of inactivated serum (AUKCᵢ), active serum (AUKCₛ), inactivated serum plus PMN (AUKCᵢ+PMN) and active serum plus PMN (AUKCₛ+PMN).

**Results:** Significant differences were found in all cases between the bactericidal activity of active serum+PMN (AUKCᵢ – AUKCₛ+PMN) and that of inactivated serum (AUKCᵢ – AUKCᵢ) with therapeutic indexes ranging from 0.56 to 3.04. At 1.5 h after dosing, a significantly higher bactericidal activity of co-amoxiclav was obtained when opsonophagocytosis occurred (samples with active serum and PMN) than when not occurring (killing curves with inactivated serum and without PMN), for all amoxicillin non-susceptible strains.

**Conclusions:** The results of this *ex vivo* study suggest that the collaboration of co-amoxiclav and complement-mediated opsonophagocytosis activated by specific antibodies may lay new approaches to overcome *in vivo* amoxicillin non-susceptibility.

Keywords: *β*-lactams, Phase I study, polymorphonuclear neutrophils, *ex vivo* killing curves

**Introduction**

The effect of antibiotics on the bacterial morphology of *Streptococcus pneumoniae* may increase phagocytosis due to the activation of the alternative complement pathway (capsular polysaccharides are not able to do this) in non-immunized hosts, or by the increase in C3 activation by immunoglobulins in the immunized host.¹ Since *β*-lactams act on the bacterial surface,
a synergic effect, with respect to bactericidal activity, with non-specific or specific immunity may be expected.\cite{2}

The effect with non-specific immunity has been demonstrated against penicillin-resistant \emph{S. pneumoniae} strains \emph{in vitro} and \emph{ex vivo} in a Phase I study with amoxicillin.\cite{4} In addition, selective binding of clavulanic acid to penicillin binding protein-3 that produces cell wall alterations\cite{5} may increase phagocytosis by (activating the alternative complement pathway),\cite{6} producing intraphagocytic death due to lysozyme action (hydrolysing peptidoglycan components) and thus, increasing global pneumococcal killing when associated with amoxicillin. In this sense, in a previous study, complement and polymorphonuclear cells (PMN) reduced the initial inocula of a serotype 9 penicillin-resistant \emph{S. pneumoniae} in the presence of physiological concentrations of clavulanic acid, but no effect was observed in the absence of clavulanic acid, or with clavulanic acid alone.\cite{7} Moreover, both PMN and clavulanic acid increased amoxicillin bactericidal activity at supra-minimal bactericidal concentrations (MBC) (achievable in serum), obtaining >90% and 99% reductions in initial inocula after only 1 and 3 h of incubation, respectively.\cite{3}

With regard to specific immunity, several animal models have demonstrated the synergic effect, with respect to the mortality end point, of passively administered specific antibodies and amoxicillin, against two penicillin-resistant serotype 6 pneumococci with amoxicillin MICs of 4 mg/L\cite{7} and 8 mg/L.\cite{8} Immunization of animals resulted in antibiotic efficacy with pharmacodynamic parameters far below the values considered necessary for efficacy: 100% survival was obtained with a time (expressed as % dosing interval) that serum levels exceed the MIC (\textgreater MIC) of 2.8% (with the strain exhibiting an MIC of 4 mg/L)\cite{7} and of 0% (with the strain exhibiting an MIC of 8 mg/L).\cite{8}

This study was carried out to investigate the effect of the presence of components of specific and non-specific immunity on co-amoxiclav bactericidal activity in humans, by measuring \emph{ex vivo} bactericidal activity (against four penicillin-resistant strains) of serum samples collected from subjects after pneumococcal immunization and a single dose administration of the pharmacokinetically enhanced co-amoxiclav sustained release 2000/125 mg formulation.

\textbf{Materials and methods}

\textbf{Subjects}

Twelve healthy male volunteers participated in this Phase I clinical trial. The study protocol was approved by the Research Ethics Committee of Hospital La Paz, Madrid. Written informed consent was obtained from all subjects before their inclusion in the study. The volunteers had the following characteristics (mean ± SD): age, 24.83 ± 5.64 years; height, 175.75 ± 7.56 cm; and weight, 73.55 ± 9.19 kg.

\textbf{Study design, drug administration and sample collection}

Volunteers received one single oral dose of the pharmacokinetically enhanced sustained release (SR) co-amoxiclav 2000/125 mg formulation (GlaxoSmithKline S.A., Madrid, Spain) with 300 mL of water. Volunteers had been fasting for 8 h before and continued for 2 h after dosing. Three days after the antibiotic dosing, the volunteers received one deep subcutaneous administration of an \emph{S. pneumoniae} polysaccharide vaccine (Aventis Pasteur S.A., Marcy l’Étoile, France). Sixty days after vaccination, the volunteers returned to the Clinical Pharmacology Unit and received a second oral single antibiotic dose. Serum samples were collected before (0 h), 1, 1.5, 2, 4, 5, 6, 8, 10 and 12 h after the first dose administration, and before (0 h), 1.5 and 5 h after the second dose administered. Serum was separated for bioassay in samples collected after the first drug administration and for bioassay and determination of bactericidal activity in samples collected after the second drug administration. Serum samples for bioassay were maintained at −70°C until assay. Peripheral polymorphonuclear neutrophils (PMN) were isolated by a differential centrifugation method\cite{9} from EDTA-treated venous blood drawn from the volunteers after the second drug administration for the \emph{ex vivo} phagocytic assay. The resulting PMN pellets were resuspended in 1 mL of Hanks’ balanced salt solution (HBSS) and held at 4°C until required.

\textbf{Ex vivo bactericidal activity}

\emph{Ex vivo} killing curves were carried out against four clinical isolates belonging to serotypes 23F, 6B, 9V and 14 and exhibiting co-amoxiclav (2:1) MIC/MBC of 2/2, 4/4, 8/8 and 16/16 mg/L, respectively. \emph{In vitro} susceptibility testing was carried out five times each by standardized methods,\cite{9} and the modal value was considered.

Bacteria in a logarithmic phase of growth were obtained by diluting an overnight culture in Todd-Hewitt broth (Difco Laboratories, Detroit, MI, USA) and placing it in a water bath (Lab-line Instruments, Melrose Park, IL, USA) until an absorbance of 0.11 at 580 nm was reached, corresponding to approximately 10^8 cfu/mL. Ex vivo killing curves were determined by placing 0.2 mL bacterial suspension, 0.2 mL PMN suspension (when appropriate) and 1.6 mL serum (collected at 0, 1.5 or 5 h, and heat-inactivated or not, as appropriate) into sterile experimental tubes containing HBSS. Killing curves were determined with HBSS (control), serum, heat-inactivated serum (inactivated serum), serum plus PMN and heat-inactivated serum plus PMN. The total volume was adjusted to 2 mL. The bacteria/PMN ratio was 10:1, and the final inoculum was approximately 10^7 cfu/mL. All tubes were simultaneously incubated at 37°C in a shaking water bath and agitated at 110 oscillations/min during the 3 h of the assay. Aliquots of 0.1 mL taken after 3 h of incubation from the experimental tubes were added to 9.9 mL of sterile water to disrupt the leucocytes and release the intracellular bacteria, thus enabling measurement of intra- and extracellular killing. After disruption, appropriate 10-fold serial dilutions were made, and 0.02 mL of each dilution was dispensed onto a blood agar plate for colony counting.

\textbf{Measurement of antibiotic concentrations in serum}

Amoxicillin and clavulanic acid concentrations were determined by bioassay. The indicator organisms were \emph{Micrococcus luteus} ATCC 9341 for amoxicillin and \emph{Klebsiella pneumoniae} NCTC 11228 for clavulanic acid. The organisms were inoculated on antibiotic agar no. 2 plates (Difco Laboratories). Each volunteer’s serum sample
was diluted in his own basal serum sample (pre-vaccination and pre-dosing) until the linearity range ($r > 0.99$): 0.06–1 mg/L for amoxicillin and 0.25–4 mg/L for clavulanic acid. A total of 0.75 mL of each sample was deposited into 6 mm diameter wells in the inoculated plates, and the plates were then incubated at 37°C for 18–24 h. Standards were prepared in pooled serum obtained from the volunteers before the administration of the drug. Standards and samples were assayed in triplicate. Limits of detection were 0.03 mg/L for amoxicillin and 0.12 mg/L for clavulanic acid. Intra-day coefficients of variation were 2.77% and 1.63%, and inter-day coefficients of variation were 0.42% and 1.45% for amoxicillin and clavulanic acid, respectively.

**Measurement of specific antibodies**

Determination of antibodies in serum samples collected pre- and post-vaccination before antibiotic administration was carried out by ELISA as previously described.

**Pharmacokinetic analysis**

Pharmacokinetic analysis was carried out by means of a model-independent approach, using the WinNonlin 2.0 professional software (Pharsight, Cary, NC, USA). Peak plasma concentration ($C_{\text{max}}$) and time to maximum plasma concentration ($T_{\text{max}}$) were determined directly from raw data. The AUCₜₐₙₜ was calculated by the trapezoidal method. Half-life ($t_{1/2}$) was calculated as ln 2/$k_{\text{el}}$. $k_{\text{el}}$ is calculated by WinNonlin from least-squares regression of the terminal phase of the curve and using at least the last three points. $t > \text{MIC}$ values for the four strains evaluated were calculated graphically after individual representation of the time–concentration profile. Therapeutic indexes (C/MIC) were individually calculated with data determined for the 12 volunteers. Comparisons of means were carried out using the $t$-test for normally distributed variables and the Signed rank test otherwise. Owing to multiple comparisons, a $P$ value < 0.005 was considered significant.

**Results**

Table 1 shows the pharmacokinetic/pharmacodynamic parameters determined with samples collected from the 12 volunteers.

<table>
<thead>
<tr>
<th>Time to maximum plasma concentration ($t_{\text{max}}$) (h)</th>
<th>1.96 ± 0.99</th>
<th>1.63 ± 0.23</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration maximum ($C_{\text{max}}$) (mg/L)</td>
<td>13.45 ± 1.81</td>
<td>1.76 ± 0.35</td>
</tr>
<tr>
<td>AUCₜₐₙₜ (mg/L × h)</td>
<td>53.36 ± 11.35</td>
<td>4.19 ± 0.89</td>
</tr>
<tr>
<td>$t &gt; \text{MIC}_{\text{serotype } 14}$</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>$t &gt; \text{MIC}_{\text{serotype } 9V}$</td>
<td>24.05 ± 6.75</td>
<td>–</td>
</tr>
<tr>
<td>$t &gt; \text{MIC}_{\text{serotype } 6B}$</td>
<td>43.26 ± 9.15</td>
<td>23.14 ± 0.50</td>
</tr>
<tr>
<td>$t &gt; \text{MIC}_{\text{serotype } 23F}$</td>
<td>56.42 ± 9.71</td>
<td>23.14 ± 0.50</td>
</tr>
</tbody>
</table>

$t > \text{MIC}$ is given as % dosing interval.

**Discussion**

In the case of β-lactams, resistance is gradual due to successive alterations in penicillin targets that make the process a concentration-dependent phenomenon. Owing to the molecular basis of pneumococcal resistance to β-lactams, one strategy to overcome it would be to increase $t > \text{MIC}$ (β-lactams are time-dependent drugs) and/or therapeutic index (concentration/MIC). Another strategy would be the study of collaboration between specific immunity and aminopenicillins (the most potent oral β-lactams regarding resistance prevalence).
There is a need to cover *S. pneumoniae* strains with amoxicillin MICs of 4 and 8 mg/L (non-susceptible strains with current NCCLS breakpoints), because since only 1% of *S. pneumoniae* have a higher MIC value, 2 coverage of these strains would result in practically 100% coverage of penicillin-resistant pneumococci, and thus of the whole pneumococcal population. A pharmacokinetically enhanced formulation of co-amoxiclav (16:1), for twice a day administration, has been developed to cover the above mentioned strains. In this study, combining both strategies, we explored the effect of specific antibodies on co-amoxiclav bactericidal activity after a single dose of the new 2000 mg SR formulation against amoxicillin non-susceptible strains with MICs of 16, 8, 4 and 2 mg/L. Mean \( t > \text{MIC} \) values determined with data from the 12 volunteers included in this study are lower than those previously reported: 24% and 43% in this study versus 35% and 49% in the previous study for strains with MICs of 8 and 4 mg/L, respectively. Mean \( t > \text{MIC} \) for the strain with an amoxicillin MIC of 2 mg/L was 56.42%, and logically 0% for the strain with an MIC of 16 mg/L, since \( C_{\text{max}} \) was 13.45 ± 8.11 mg/L.

The bactericidal activity was explored at approximately peak concentrations and at 5 h post-dosing, representing around 40% of the dosing interval, the \( t > \text{MIC} \) value needed for penicillin efficacy. Bactericidal activity was explored by studying the effect of the presence/absence of components of the immune system (subtracting or not subtracting complement activity by heat inactivation, and by adding or not adding PMN) in the serum samples that always contained antibiotic concentrations and specific antibodies. The effect was investigated by measuring the reduction in the growth curve produced by the presence of antibiotic concentrations with and without different components of the immune system, by determining the difference between AUKC\( _K \) (control growth curve) and the different sample AUKCs (AUKC\( _{\text{IS}} \), AUKC\( _{\text{IS}+\text{PMN}} \), AUKC\( _{\text{S}} \) or AUKC\( _{\text{S}+\text{PMN}} \)). AUKC was used as a measure of global killing over 3 h as a more accurate measure of the overall exposure effect than simply the serum bactericidal activity. In addition, it is the way to measure the bactericidal activity in the presence of PMN with the limitation of their 3 h viability.

The immune system attacks *S. pneumoniae* by opsonophagocytosis mediated by complement activated by specific antibodies. This study explores the effect of the presence of supra- and sub-inhibitory amoxicillin concentrations, and the deletions of PMN and/or complement activity in this process. At peak concentrations, mean differences of areas are positive in all cases, showing that the AUKC\( _K – \text{AUKC}_\text{IS} \) (growth curve) is always reduced. This occurs even at sub-inhibitory concentrations (strain exhibiting MIC of 16 mg/L and a therapeutic index of 0.76). Against this strain, sub-inhibitory antibiotic concentrations have no effect in the absence of PMN and complement activity (AUKC\( _K – \text{AUKC}_\text{IS} = 0.18 \log \text{cfu} / \text{h/mL} \)) whereas a significantly different activity is obtained when adding complement and PMN (AUKC\( _K – \text{AUKC}_\text{S+PMN} = 0.76 \pm 0.54 \log \text{cfu} / \text{h/mL} \)). These significant differences when complement and PMN were present are maintained with strains with MICs of 8 and 4 mg/L (1.59 ± 0.67 and 2.81 ± 0.79, respectively), but not with the strain

Table 2. Therapeutic index (serum concentration/MIC), and serum bactericidal activity measured as differences in AUKCs (log cfu × h/mL) of control (K) and inactive serum (IS), IS + PMN, active serum (S) and S + PMN, at 1.5 h after dosing (means ± SD)

<table>
<thead>
<tr>
<th>MIC (mg/L)</th>
<th>Serotype 14</th>
<th>Serotype 9V</th>
<th>Serotype 6B</th>
<th>Serotype 23F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Therapeutic index</td>
<td>0.76 ± 0.19</td>
<td>1.52 ± 0.39</td>
<td>3.04 ± 0.77</td>
<td>6.08 ± 1.55</td>
</tr>
<tr>
<td>AUKC( <em>K – \text{AUKC}</em>\text{IS} )</td>
<td>0.18 ± 0.82</td>
<td>0.83 ± 0.64</td>
<td>2.22 ± 0.82</td>
<td>3.68 ± 0.62</td>
</tr>
<tr>
<td>AUKC( <em>K – \text{AUKC}</em>\text{IS+PMN} )</td>
<td>0.24 ± 0.82</td>
<td>0.80 ± 0.66</td>
<td>2.26 ± 0.87</td>
<td>3.77 ± 0.80</td>
</tr>
<tr>
<td>AUKC( <em>K – \text{AUKC}</em>\text{S} )</td>
<td>0.54 ± 0.47</td>
<td>1.49 ± 0.65*</td>
<td>2.63 ± 0.80*</td>
<td>4.01 ± 0.67</td>
</tr>
<tr>
<td>AUKC( <em>K – \text{AUKC}</em>\text{S+PMN} )</td>
<td>0.76 ± 0.54*</td>
<td>1.59 ± 0.67*</td>
<td>2.81 ± 0.79*</td>
<td>4.21 ± 0.67</td>
</tr>
</tbody>
</table>

*Statistical differences versus AUKC\( _K – \text{AUKC}_\text{IS} \) (P < 0.005).

Table 3. Therapeutic index (serum concentration/MIC), and serum bactericidal activity measured as differences in AUKCs (log cfu × h/mL) of control (K) and inactive serum (IS), IS + PMN, active serum (S) and S + PMN, at 5 h after dosing (means ± SD)

<table>
<thead>
<tr>
<th>MIC (mg/L)</th>
<th>Serotype 14</th>
<th>Serotype 9V</th>
<th>Serotype 6B</th>
<th>Serotype 23F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Therapeutic index</td>
<td>0.28 ± 0.10</td>
<td>0.56 ± 0.20</td>
<td>1.13 ± 0.40</td>
<td>2.26 ± 0.82</td>
</tr>
<tr>
<td>AUKC( <em>K – \text{AUKC}</em>\text{IS} )</td>
<td>−0.41 ± 0.71</td>
<td>−0.40 ± 0.57</td>
<td>0.25 ± 0.81</td>
<td>2.22 ± 1.20</td>
</tr>
<tr>
<td>AUKC( <em>K – \text{AUKC}</em>\text{IS+PMN} )</td>
<td>−0.57 ± 0.69</td>
<td>−0.40 ± 0.52</td>
<td>0.53 ± 0.78</td>
<td>2.43 ± 1.22</td>
</tr>
<tr>
<td>AUKC( <em>K – \text{AUKC}</em>\text{S} )</td>
<td>−0.35 ± 0.58</td>
<td>−0.12 ± 0.51</td>
<td>1.08 ± 0.93*</td>
<td>2.89 ± 1.12*</td>
</tr>
<tr>
<td>AUKC( <em>K – \text{AUKC}</em>\text{S+PMN} )</td>
<td>−0.20 ± 0.67</td>
<td>−0.01 ± 0.77*</td>
<td>1.32 ± 0.87*</td>
<td>3.13 ± 1.19*</td>
</tr>
</tbody>
</table>

*Statistical differences versus AUKC\( _K – \text{AUKC}_\text{IS} \) (P < 0.005).
with an MIC of 2 mg/L, where the activity of the antibiotic alone is very high (AUKC<sub>K</sub>−AUKC<sub>IS</sub> = 3.68 ± 0.62 log cfu·h/mL). At 5 h, significant differences were found with strains with MICs of 8, 4 and 2 mg/L. In both cases (at 1.5 and 5 h), significant differences by adding complement and PMN to the antibiotic–antibody mixture occur with therapeutic index values between 0.76 and 3.04. Very high values (strain with MIC of 2 mg/L at 1.5 h; high activity of the antibiotic alone) or very low values (strain with MIC of 16 mg/L at 5 h) precluded these differences.

When comparing AUKC<sub>K</sub>−AUKC<sub>S</sub> values versus AUKC<sub>K</sub>−AUKC<sub>IS</sub>, that is, when exploring bacterial death by interactions of the antibiotic and the killing mediated by complement activated by specific antibodies (absence of PMN), a significant activity in the collaboration of the antibiotic and the immune system is observed in a more narrow range of therapeutic indexes (1.13–3.04). This mechanism of collaboration is normally hidden by the collaboration between the antibiotic and the opsonophagocytosis mediated by antibody complement activation which, as shown in Tables 2 and 3, is more effective (higher values for AUKC<sub>K</sub>−AUKC<sub>S</sub>−PMN than for AUKC<sub>K</sub>−AUKC<sub>S</sub>). In this study, it was shown that an increase in the global killing by co-amoxiclav occurred over 3 h by opsonophagocytosis mediated by complement activated by specific antibodies, or to a lesser extent, by killing due to complement activated by specific antibodies (which may be important in cases of neutropenia). This effect was shown using as control the activity of the antibiotic alone. The results of this study may underestimate the antibiotic activity because, in the tubes used for ex vivo killing curves, serum represented 80% fraction (1.6 mL in 2 mL final volume), and thus, 80% of in vivo therapeutic indexes. On the other hand, in this study, no methods for capsular enrichment (as animal passage and enriched media) were carried out with the study strains. Results may also be underestimated for this reason, since increases in the capsule increase opsonophagocytosis activity because the capsule is the target of specific antibodies. Despite these ex vivo underestimations of both the antibiotic and the immune system in this Phase I study, the results (with the end point of bacterial killing over 3 h) suggest that the collaboration of both antibacterial weapons (the physiological one—the immune system, and the pharmacological one—co-amoxiclav SR) may set strategies to overcome in vivo the amoxicillin non-susceptibility. The effectiveness of this strategy, and the translation of the ex vivo facts described in this study (enhanced β-lactam bacterial killing by opsonophagocytosis in the presence of specific antibodies), have been shown in animal models with a different end point (survival over 7 days).^8

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References


