β-Lactam modification of the bacteraemic profile and its relationship with mortality in a pneumococcal mouse sepsis model

J. Yuste\textsuperscript{a}, I. Jado\textsuperscript{a}, A. Fenoll\textsuperscript{a}, L. Aguilar\textsuperscript{b}, M. J. Giménez\textsuperscript{b} and J. Casal\textsuperscript{a*}

\textsuperscript{a}Centro Nacional de Microbiología, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo, Km. 2, 28220 Majadahonda, Madrid; \textsuperscript{b}Medical Department, GlaxoSmithkline, Tres Cantos, Madrid, Spain

A sepsis BALB/c mice model was used to investigate the relationship between mortality and the bacteraemic profile produced by a serotype 6B \textit{Streptococcus pneumoniae} clinical isolate (MIC/MBC of amoxicillin 4/4 mg/L and of cefotaxime 2/4 mg/L). Animals were treated subcutaneously with doses of amoxicillin or cefotaxime ranging from 6.25 to 50 mg/kg tds for 48 h, starting 1 h after intraperitoneal inoculation (2 × 10\textsuperscript{7} cfu/mouse). Blood cultures were carried out daily over 15 days. A survival rate of 100% was obtained with amoxicillin 25 mg/kg and of 60% with cefotaxime 50 mg/kg. A statistically significant (\(P = 0.012\)) relationship was found between the maximum cfu/mL in blood and mortality. A maximum log cfu/mL of 6.5 was associated with an 84% probability of death.

Introduction

Two parameters are commonly used for the evaluation of antibiotic efficacy in animal models: bacterial counts in tissue fluids and survival rate.\textsuperscript{1} The second parameter is easy to assess and seems more relevant from the clinical perspective; a curative dose is used as a reliable end-point for showing subtle differences in antibiotic behaviour.\textsuperscript{2} Reduction in bacterial counts provides information on bactericidal activity \textit{in vivo};\textsuperscript{1} nevertheless, \textit{in vitro} inocula and an inocula reduction rate equivalent to those \textit{in vivo} have never been established\textsuperscript{3} and reduction in bacterial count may not be related to animal survival.\textsuperscript{2}

Bacterial virulence is the minimal bacterial mass capable of producing injury to a given host\textsuperscript{4} and can be defined as the competence of an infectious agent to produce pathological effects, as indicated by case fatality rates and/or the ability to invade the host.\textsuperscript{5} This study explores the modification by β-lactam therapy of the bacteraemic profile (representative of ability to invade the host) produced by a bacterial mass (representative of virulence) and its relationship with mortality rates (representative of competence to produce pathological effects). A serotype 6 penicillin-resistant isolate of \textit{Streptococcus pneumoniae} was used as infecting strain, as this is one of the most frequently isolated serotypes in bacteraemia and respiratory-tract infections in Spain.\textsuperscript{6,7}

Materials and methods

\textbf{Infecting strain}

A serotype 6B \textit{S. pneumoniae} strain isolated from a blood culture [MIC and minimum bactericidal concentration (MBC) of penicillin 2 and 4 mg/L, respectively] was used. The microorganism was grown until an absorbance of 0.3 (UV-VIS spectrophotometer, Shimadzu UV-1203, Japan) was obtained in Todd–Hewit broth supplemented with 0.5% yeast extract (THYB) (Difco, Detroit, MI, USA), and aliquots were stored at \(-70^\circ\text{C}\) in 15% glycerol.

\textbf{In vitro studies}

MICs and MBCs of amoxicillin and cefotaxime were determined by broth dilution following NCCLS procedures.\textsuperscript{8} Modal values of five separate determinations were considered.

\textbf{Animals}

Eight- to 12-week-old female BALB/c mice weighing 19–22 g were used. The study was approved by the Spanish Central Laboratory of Public Health—Instituto de Salud Carlos III.

*Corresponding author. Tel: +34-91-509-7975; Fax: +34-91-509-7966; E-mail: jcasal@isciii.es

© 2002 The British Society for Antimicrobial Chemotherapy
**Determination of minimal lethal dose**

Groups of 10 mice per dilution were injected intraperitoneally with 0.2 mL of different inocula, 10^2, 10^4, 10^6 and 10^8 cfu/mL (spectrometrically measured), to determine the minimal dose that produced a 100% mortality over a 15 day follow-up period (minimal lethal dose, MLD). Bacteria in a logarithmic phase of growth in THYB were centrifuged and the pellets washed three times and resuspended in PBS pH 7.2 to reach the desired turbidity. The inoculum was confirmed by the culture of serial dilutions onto blood Mueller–Hinton agar incubated at 37°C in 5% CO₂ air. Mouse mortality was recorded daily. The MLD was determined from the results obtained in three independent experiments.

**Dose-ranging treatment**

Survival and the bacteraemic profile of inoculated animals over a 15 day follow-up period were determined in a dose-ranging study with amoxicillin and cefotaxime doses ranging from 6.25 to 50 mg/kg. Animals were inoculated intraperitoneally with 200 μL of the MLD, and antibiotic treatment was initiated 1 h after bacterial inoculation. Groups of five animals per dose were treated with 100 μL subcutaneously tds for 48 h. The five animals of the control group received placebo (apyrogen sterile distilled water). Animals were observed and deaths were recorded for 15 days.

Blood samples were obtained daily (except on day 1 when they were collected at 2, 6 and 24 h) over the 15 day follow-up period, from five animals per antibiotic dose, to study the bacteraemic profile. Tails were disinfected and anaesthetized, and the end portion of the tail was amputated with scissors. Using a calibrated loop, 0.008 mL of blood were taken and resuspended in Todd–Hewitt broth containing 0.012–1.6 and 0.4–50 mg/L were prepared in apyrogen distilled water for amoxicillin and cefotaxime, respectively, to determine the assay regression line (standard curve) and to extrapolate the antibiotic concentrations from the corresponding inhibition zone diameters.

**Pharmacokinetic study**

Concentration–time curves for each antibiotic were analysed by a non-compartmental approach using the WinNonlin program (Pharsight, Mountainview, CA, USA). The areas under the serum concentration–time curves 0 h to ∞ (AUC₀–∞) were calculated from the equation

\[ \text{AUC}_0-\infty = \text{AUC}_0-480\text{min} + \text{AUC}_{480\text{min}}-\infty \]

The values for AUC₀–480min were calculated from plots of serum concentrations versus time by using the trapezoidal rule. The values for AUC₄₈₀ₗₘ₈ were calculated from the expression

\[ \text{AUC}_{480\text{min}}-\infty = \text{C}_{\text{max}}/\beta \]

where β is the slope obtained from least-square regression of the terminal elimination phase. The value of β was calculated for each antibiotic using at least the last three sample time values of serum concentrations. The theoretical concentration at time 0 (obtained by back-extrapolation to the origin of the elimination regression line) was considered the maximum concentration in serum (Cₘₐₓ). Time above MIC (ΔT > MIC) was calculated graphically from the semi-logarithmic plot representing the concentration–time data.

**Statistical analysis**

Survival curves were obtained by the Kaplan–Meier method. A dose-adjusted Cox regression analysis was used to compare survival with each antibiotic. A Probit regression analysis was carried out to calculate the 50% efficacy dose (EC₅₀) in the theoretical model using the dose as covariable and the antibiotic as factor. A binary logistic regression analysis was used to study the relationship between the maximum cfu/mL and outcome (death/survival) per individual.

**Results and discussion**

Modal MIC/MBC values of amoxicillin and cefotaxime were 4/4 and 2/4 mg/L, respectively, for the infecting strain. Considering the MIC₉₀ of both drugs determined in previous studies in Spain, the susceptibility of the strain used in the present study represents the resistance pattern in this country. The MLD was 10^6 cfu/mL (i.e. 2 × 10⁵ cfu/mouse). Since 10^6 cfu/mL was the minimal bacterial mass producing 100% mortality, this value could be considered representative of the virulence of this type 6B strain. When evaluating...
Pneumococcal bacteraemia and mortality with β-lactams

the competence of this MLD to produce pathological effects through the ability to invade the host, by studying the bacteraemic profile, it was observed that the mean blood colony counts in untreated animals were \( \geq 10^7 \) cfu/mL at 2, 6 and 24 h sample times (Figures 1 and 2), with all untreated animals having bacterial counts \( > 10^6 \) cfu/mL until death.

The Table shows percentages of survival with the differ-

Figure 1. Bacteraemic profiles (blood colony counts over 360 h) of animals treated with amoxicillin: ■, inoculum; □, control; +, 6.25 mg/kg; ◆, 12.5 mg/kg (death); *, 12.5 mg/kg (survival); ▲, 25 mg/kg; ○, 50 mg/kg.

Figure 2. Bacteraemic profiles (blood colony counts over 360 h) of animals treated with cefotaxime: ■, inoculum; □, control; +, 6.25 mg/kg; ◆, 12.5 mg/kg; ▲, 25 mg/kg; ○, 50 mg/kg (death); ○, 50 mg/kg (survival).

333
ent antibiotic regimens over the first 144 h of the follow-up period. Survival percentages at 360 h were identical to those at 144 h. The mean survival time obtained for amoxicillin was greater than that obtained for cefotaxime at each dose (mean days for amoxicillin versus cefotaxime: 2.1 vs 1.7 for 6.25 mg/kg, 8.7 vs 2.9 for 12.5 mg/kg, >15 vs 2.9 for 25 mg/kg and >15 vs 11 for 50 mg/kg). Statistically significant ($P = 0.014$) differences were found between both antibiotics in survival curves. The dose obtaining EC 50 in the theoretical model was 12.6 mg/kg for amoxicillin and 47.5 mg/kg for cefotaxime; the median relative efficacy between antibiotics was 0.2657 favouring amoxicillin.

The $C_{\text{max}}$ and the AUC were, respectively, 270.6 mg/L and 7657.7 mg/min/L for amoxicillin 25 mg/kg and 621.4 mg/L and 16483.8 mg/min/L for cefotaxime 50 mg/kg. The time that serum concentrations were higher than the MIC ($T >$ MIC) was 123 min for amoxicillin 25 mg/kg and 156 min for cefotaxime 50 mg/kg, representing 26% and 32% of the dosing interval, respectively. Previous studies have shown that $T >$ MIC of 40% of the dosing interval is needed for $\beta$-lactams to obtain therapeutic efficacy. $^9$ Percentages are lower for penicillins than for cephalosporins (reflecting the fastest killing with penicillins);$^9$ this fact is corroborated in the present study, since 100% survival was obtained with $T >$ MIC of 26% for amoxicillin 25 mg/kg but not with $T >$ MIC of 32% for cefotaxime 50 mg/kg.

Figures 1 and 2 show the mean bacteraemic profile (blood culture cfu/mL over 360 h) with the different amoxicillin and cefotaxime doses, with animals divided in each study group by outcome: death and survival. As can be seen, mean blood colony counts in surviving mice were $\leq 10^6$ cfu/mL over the 360 h. In contrast, maintained mean bacteraemic colony counts $> 10^6$ cfu/mL over the first 144 h were found in those animals that died. This can be clearly seen with the amoxicillin 12.5 mg/kg and cefotaxime 50 mg/kg regimens, where some animals survived and some died, and where both bacteraemic profiles were present. A statistically significant ($P = 0.012$) relationship was found between the maximum cfu/mL in blood and mortality, with $\beta_0 = -15.21$ and $\beta_1 = 2.5944$ as logistic regression coefficients. In the theoretical model, a maximum log cfu/mL of 6.5 was associated with an 84% probability of death.

In contrast to previous studies, where pneumococcal counts in blood were poorly related to the outcome of infection in penicillin-treated animals,$^{10}$ in the present study, using a penicillin-resistant strain, bacterial counts were good indicators of mouse survival and death. The two $\beta$-lactams (amoxicillin to a higher degree) reduced fatality rates of the serotype 6B pneumococcal strain by decreasing colony counts of the bacteraemic profile.

### Acknowledgements

We thank J. Prieto and M. L. Gómez-Lus (Universidad Complutense, Madrid, Spain) for their technical advice and critical review of the manuscript, A. Carcas (Universidad Autónoma, Madrid) for the pharmacokinetic analysis, A. Pedromingo (www.e-biometria.com) for the statistical analysis and F. Molero (Centro Nacional de Microbiología. Instituto de Salud Carlos III) for her technical assistance. This study was supported by European Funds for Regional Development and by funds from the Spanish National R+D Program (Project 2FD 97-0554).

### References

Pneumococcal bacteraemia and mortality with β-lactams


Received 15 June 2001; returned 25 September 2001; revised 17 October 2001; accepted 12 November 2001