An *in Vitro* Pharmacodynamic Model to Simulate Antibiotic Behavior of Acute Otitis Media with Effusion

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The purpose of this investigation was to develop an *in vitro* pharmacodynamic model (IVPM) that would simultaneously simulate *in vivo* serum and middle ear amoxicillin pharmacokinetic characteristics of acute (purulent) otitis media and then utilize the IVPM to assess amoxicillin-mediated killing of a type 7F *Streptococcus pneumoniae* (MIC = 0.002 mg/L). The IVPM consisted of a sterile central compartment and a membrane-bound "infected" peripheral compartment. Peak peripheral compartment amoxicillin concentrations occurred within 2 hr after its introduction into the central compartment and were approximately 30% of peak central compartment concentrations. Amoxicillin elimination from the central compartment was designed to provide a 1-hr $t_{1/2}$. Amoxicillin elimination from the peripheral compartment was slower than from the central compartment, with an average half-life of 2.3 hr. Significant concentration-related differences in maximal bacterial kill rates were not detected over the range of amoxicillin concentrations studied (0.26 to 14.6 mg/L). However, at peak central compartment amoxicillin concentrations of ≤2 mg/L, a lag phase in killing was observed. In general, the *in vitro* pharmacokinetic data derived from this model compare well with published *in vivo* data.

KEY WORDS: *in vitro;* pharmacodynamics; pharmacokinetics; amoxicillin; otitis media.

INTRODUCTION

Otitis media with effusion (OME) is a disease that occurs frequently in the pediatric population (1). An estimated 30 million visits to physicians offices per year are attributed to this disease at an annual cost exceeding 1 billion dollars (2). Despite the prevalence of this disease, there exists a great deal of controversy regarding appropriate management including drug selection, treatment duration, and approach to treatment failure (3). Bacteria have been implicated as the etiologic agents in the majority of OME cases. *Streptococcus pneumoniae* has been cultured in 25–50% of acute, middle ear effusions in children 1 year of age and older, and *Haemophilus influenzae* has been found in 15–20% of these children (4).

Among children unsuccessfully treated with antibiotic therapy, only 20% have a resistant bacterial pathogen isolated from the middle ear (5). This suggests that treatment failure may be due to the consequences of inadequate antibiotic penetration to the infected middle ear site. The primary goal of this study was to develop an *in vitro* pharmacodynamic model (IVPM) that simulates *in vivo* antibiotic pharmacokinetics of acute OME with respect to the serum and middle ear. This IVPM was developed to provide a precise dynamic methodology for the controlled study of various infectious disease processes associated with otitis media.

MATERIALS AND METHODS

The *in vitro* model design was similar to that previously published by Shah (6). The critical difference between the model described here and models described previously is the exact ratio of the surface membrane area to volume of the peripheral compartment (0.76 cm^{-1}), which, together with other variables described by Fick's law of diffusion, determines antibiotic pharmacokinetic parameters relative to the peripheral compartment.

The *in vitro* model was composed of a glass central compartment and a glass peripheral compartment (Fig. 1). The central compartment volume was 285 ml and the peripheral compartment volume was 6 ml. The peripheral compartment was a hollow "T" tube fitted with an inert polycarbonate, 0.4-μm-pore size membrane (Model PL-Memb-47mm, Serial No. 111107, Nucleopore Corp., Pleasanton, CA) on each end. The central compartment and peripheral compartment were continually agitated by separate magnetic stir bars to provide thorough mixing. Although bacteria could not penetrate through the membranes, antibiotic and growth medium components could freely pass. By diffusion, the growth medium in the central compartment penetrated and equilibrated with the peripheral compartment.

All experiments were contained in a stirred water bath with the temperature maintained at 37°C. Sterility of all experimental components, except connecting tubing, was accomplished by autoclaving (121°C, 15 psi for 30 min). Connecting tubing was gas sterilized with ethylene oxide.

At the beginning of each experiment amoxicillin was bolus injected into the central compartment to achieve a range of predetermined peak concentrations of from approximately 0.3 to 15 mg/L. A total of 13 experiments was performed, with peak amoxicillin central-compartment concentrations ranging from (at time = 0 hr postbolus by extrapolation) 0.26 to 14.6 mg/L. This range of central-compartment amoxicillin concentrations corresponded to a range of peak peripheral-compartment concentrations (0.07 to 5 mg/L) which was consistent with middle ear effusion amoxicillin concentrations observed in children following a single standard oral dose (13 mg/kg) of amoxicillin (7). A central compartment amoxicillin half-life of 1 hr was simulated for all experiments. Todd-Hewitt (BBL Microbiology Systems, Becton-Dickinson and Co., Cockeysville, Maryland) medium containing antibiotic was displaced from the central

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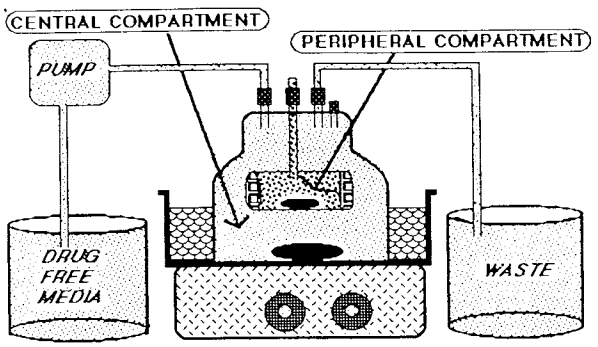

Fig. 1. A schematic of the *in vitro* pharmacodynamic model (reprinted with permission from *Antimicrob. Agents Chemother.* 34:1925–1931 (1990).

compartment at the same rate (3.2 ml/min) that antibiotic-free growth medium was pumped into the central compartment using a peristaltic pump (Masterflex, Cole-Palmer, Chicago, IL). This displacement was designed to simulate a first-order pharmacokinetic clearance process from the central compartment. The duration of each experiment was 4 hr. Medium samples (100 μl) were taken from the central and peripheral compartments every 30 min.

Type 7F *S. pneumoniae* was selected for study because it has been identified as a virulent cause of OME in animals (8,9). Prior to the beginning of each experiment, several *S. pneumoniae* colonies (clinical isolate, MIC = 0.002 mg/L) were picked from a fresh stock plate (<24 hr old) with a sterile loop and placed in 3 ml of warmed Todd-Hewitt broth. This culture was then incubated (VWR Scientific incubator, VWR 6000, San Francisco, CA) for a minimum of 3 hr (at 37°C, 5% CO_2, and 2 L/min of air) to ensure exponential growth. The optical density of the culture was determined at 380 nm using a spectrophotometer (Shimadzu Corp., Model UV-160, Kyoto, Japan). An appropriate dilution of the culture was chosen to yield a starting inoculum of approximately 10^7 cfu/ml. From a previously developed standard growth absorbance curve specific for this organism, this starting inoculum was chosen because Wald *et al.* (10) have associated a middle ear effusion inoculum of $>10^4$ cfu/ml with treatment failures. The peripheral compartment was inoculated with this exponentially growing culture 30 min prior to the start of each experiment.

Peripheral compartment samples were assayed for cfu/ml of *S. pneumoniae* using six or seven serial dilutions of the sample aliquot with sterile Todd Hewitt medium as the diluent. Only those peripheral compartment samples that were diluted 1:20 or greater were used for kill curve analysis to prevent an antibiotic carryover effect. To prevent a significant volume reduction and subsequent dilution of the peripheral compartment, medium sampling for amoxicillin and bacterial concentration determinations was confined to 100 μl per sample. This resulted in a lower limit of accurately detectable bacterial burdens of 6×10^3 cfu/ml. Aliquots of each dilution (100 μl) were immediately plated onto blood agar plates (trypticase soy agar with 5% sheep blood; BBL) and incubated for 18 hr at 37°C, 5% CO_2, and 2 L/min of air. After incubation each plate was inspected for bacterial growth. Only those plates with >30 and <300 bacterial cfu were used for analysis.

To validate sterility of the central compartment, duplicate 100-μl samples from the central compartment were plated on blood agar plates and incubated for 18 hr (37°C, 5% CO_2, 2 L/min of air). Recovery of any bacteria was considered evidence that the experiment had been contaminated, however, there were no experimental failures resulting from contamination.

Assay

Micrococcus lutea (ATCC 9341) was used as the reference organism for the microbiological diffusion assay determination of amoxicillin concentrations. On the day prior to each experiment, antibiotic assay medium 1 (Difco Laboratories, Detroit, MI) at 52°C was mixed with a standard suspension of *M. lutea* and pipetted onto sterile petri dishes in 8-ml aliquots. After cooling to 5°C, 6-mm wells were cut in these assay plates. The plates were stored overnight at 5°C. Amoxicillin (Amoxil, Lot 23086A, Bencard, Brentford, England) stock solution was prepared on the day of the experiment. The stock solution was serially diluted to give amoxicillin concentrations ranging from 0.01 to 30 mg/L, and a standard curve was prepared by placing 20-μl aliquots of each concentration into the assay plate wells. The assay plates were incubated at 37°C for 18 hr. Each sample was assayed in duplicate, with two perpendicular readings per well per plate. The zones of inhibition were read to the nearest 0.5 mm. The results of the four readings at each concentration were averaged. The standard curve for determination of amoxicillin concentrations in experimental samples was constructed by graphing log-linearly the concentration of amoxicillin versus the zone of inhibition in centimeters. Amoxicillin concentrations ranging from 0.02 to 30 mg/L could be measured reliably (intra- and interday CV, ≤3.4%). A new standard curve was constructed each day an experiment was done. All experimental samples assayed for amox-

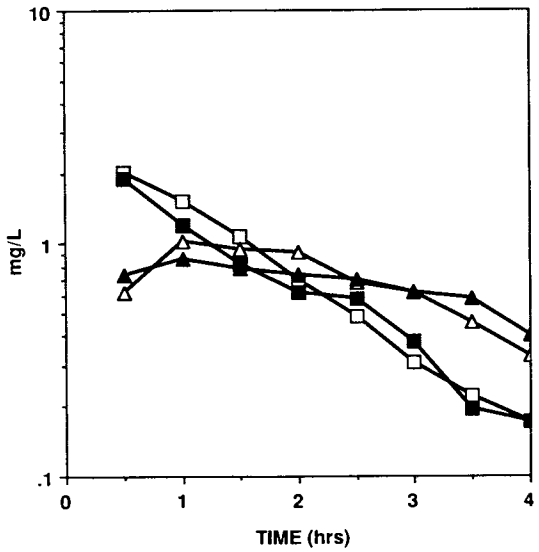

Fig. 2. A log-linear graph of central compartment (CC) and peripheral compartment (PC) amoxicillin concentrations over time for duplicate experiments. (□) CC, Expt 1; (■) CC, Expt 2; (△) PC, Expt 1; (▲) PC, Expt 2.

icillin concentration were analyzed immediately after extraction from the model.

Data Analysis

Amoxicillin concentration (cfu/ml) versus time data were graphed in a log-linear fashion to describe the *in vitro* pharmacokinetics. For each experiment, least-squares linear regression analysis was performed to determine (i) the amount of time required for a 50 and 99% kill of the initial inocula and (ii) the maximal killing rate, given by the slope of the killing curve (hr^{-1}). Results are reported as means ± 1 SD.

RESULTS

When central-compartment amoxicillin concentration versus time data were graphed log-linearly, first-order elimination of amoxicillin was observed (Fig. 2). Linear regression analysis of these data resulted in correlation coefficient values ≥0.99 for all experiments. The experimentally determined central-compartment amoxicillin half-life was 0.97 ± 0.08 hr and the peripheral-compartment half-life was 2.33 ± 0.60 hr. Peak peripheral-compartment concentrations were achieved within 1 to 2 hr after the amoxicillin dose. Peak peripheral compartment-to-peak central compartment amoxicillin concentration ratios ranged from 0.23 to 0.34.

Figure 3 summarizes all experimental *S. pneumoniae* kill curves with amoxicillin, including a control growth curve where no antibiotic was present. Linear regression analysis of log-linear graphs of all kill curves resulted in correlation coefficients ranging from 0.94 to 1.0. A similar rapid bactericidal effect was observed in all experiments at all concentrations of amoxicillin studied, despite an approximately 50-fold variation in the peak central compartment concentration of amoxicillin. The maximum killing rate given by the slope of the killing curve (hr^{-1}) remained similar at all concentrations of antibiotic studied (1.84 ± 0.30).

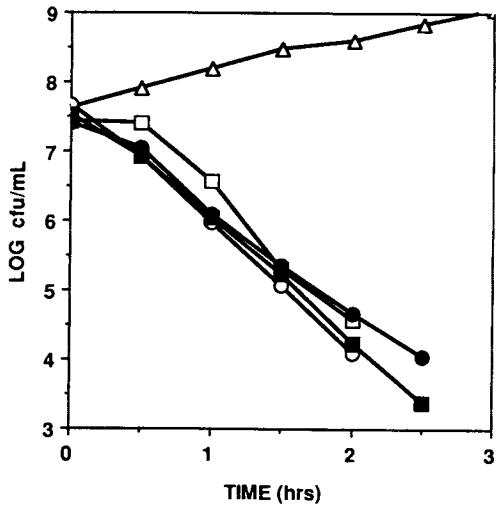

Fig. 3. A log-linear graph of the number of cfu/ml of *S. pneumoniae* over time after exposure to varying central-compartment peak concentrations of amoxicillin. Each data point at a given concentration is an average value for two or more experiments. Amoxicillin concentrations: (△) 0 mg/L; (□) 0.3 mg/L; (●) 3 mg/L; (○) 6 mg/L; (■) 10 mg/L.

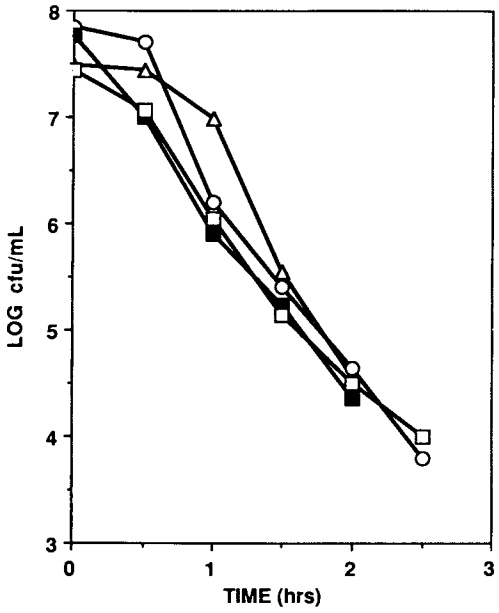

Fig. 4. A log-linear graph of the number of cfu/ml of *S. pneumoniae* over time after exposure to varying peak central-compartment concentrations of amoxicillin demonstrating a lag phase in killing at amoxicillin concentrations <2 mg/L. Amoxicillin concentrations: (△) 0.3 mg/L; (○) 0.5 mg/L; (■) 2 mg/L; (□) 3 mg/L.

A lag phase in killing was observed as the peak central-compartment amoxicillin concentrations were decreased to <2 mg/L (Fig. 4). The time to reach a 50% kill of the initial inoculum increased overall as the amoxicillin concentration decreased. However, the time to reach a 99% kill was similar at all concentrations of amoxicillin (1.36 ± 0.13 hr) (Table I).

DISCUSSION

A key tool used by the clinician in selecting appropriate antimicrobial therapy is *in vitro* minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) susceptibility testing. Universally accepted standards have been established for performing and reporting the results of

Table I. *In Vitro* Lag Phase Killing Described in Relationship to the Time Required to Reach a 50 and a 99% Kill of the Initial *S. pneumoniae* Inoculum[a]

Average peak amoxicillin concentration in the central compartment (mg/L)	Time to a 50% kill (hr)	Time to a 99% kill (hr)
0.3	0.75	1.48
0.5	0.44	1.35
2.0	0.16	1.17
3.0	0.38	1.53
6.0	0.25	1.28
10.0	0.32	1.34

[a] n = 12 experiments, 2 per group.

Table II. A Comparison of Amoxicillin Pharmacokinetic Data Derived from *In Vitro*, Chinchilla, and Human OME Studies

Source of data	Central-compartment or serum $t_{1/2}$ (hr)	Peripheral-compartment or middle ear effusion $t_{1/2}$ (hr)	Peak peripheral compartment-to-peak central compartment concentration ratio	Time to peak peripheral-compartment concentration (hr)
In vitro Purulent OME modeling	0.97	2.33	0.30	1–2
Chinchilla purulent OME modeling (14)	0.90	1.6	0.378	1–2
Human mixed-type (purulent, serous, and mucoid) OME data (7)	1.39[a]	3.96[a]	0.125	1–2

[a] Due to the experimental design of the human study, the human data presented represent global estimates of pharmacokinetic parameters, and not precise values.

these tests. Because these tests are done by exposing bacteria over long periods of time to static concentrations of antibiotic, *in vitro* testing is not representative of *in vivo* conditions where antibiotic concentrations are in a dynamic state of flux. The pharmacokinetic discrepancy between susceptibility *in vitro* testing and the *in vivo* situation is magnified with antibiotics with short serum half-lives such as amoxicillin. Additionally, traditional *in vitro* susceptibility testing is not designed to assess the impact of variables such as inoculum magnitude, pH, ion content, and protein binding on antibiotic pharmacodynamics. Because of these confounding variables, new approaches to *in vitro* investigations with application to specific infectious diseases need to be developed.

A prerequisite to an accurate *in vitro* assessment of the interaction that occurs in the middle ear when pathogens are exposed to antibiotic is the ability to simulate simultaneously *in vivo* antibiotic concentrations in a central (e.g., serum) and a peripheral (e.g., middle ear) compartment. The data reported herein compare well with animal and human studies in that (Table II): (i) similar central-compartment antibiotic half-lives were observed (11–13); (ii) peripheral-compartment antibiotic half-lives were longer than those of the central compartment (7,14,15); (iii) peak peripheral-compartment antibiotic concentrations were approximately 30% of central compartment values (7,14,15); and (iv) the time to reach peak peripheral-compartment antibiotic concentrations was 1 to 2 hr (7,14). Our *in vitro* amoxicillin pharmacokinetic data compare less favorably to the human OME data (7) than to the chinchilla OME data. This was not an unexpected finding. The *in vitro* model as described in this report was designed to simulate amoxicillin pharmacokinetics in the setting of acute (purulent) OME and not chronic (serous or mucoid) OME. Very few human data have been published in this area. The investigation by Kim *et al.* (7) represents pooled data, most of which are from patients with chronic OME. In contrast, Canafax *et al.* (14) describe animal amoxicillin pharmacokinetic data in the setting of Type 7F *S. pneumoniae*-induced acute OME.

Despite an approximately 50-fold change in amoxicillin concentrations, the *S. pneumoniae* kill curves were almost superimposable. These results might be explained by considering that even at the lowest peak concentration of amoxicillin observed in the peripheral compartment (0.07 mg/L), the ratio of this concentration to the minimum inhibitory concentration of amoxicillin for *S. pneumoniae* (0.002 mg/L) was still extremely large.

The lag phase observed in bacterial killing was amoxicillin concentration dependent. This is probably a reflection of the time required to achieve a threshold bactericidal concentration of amoxicillin in the peripheral compartment. Fick's law would suggest that the lower the peak central compartment antibiotic concentration, the longer the time required to achieve a given peripheral compartment concentration (18). The threshold concentration probably represents some ratio of the achieved amoxicillin concentration versus the minimum inhibitory concentration. Once that concentration of antibiotic is achieved, a maximal rate of bacterial killing is observed. The concept of a threshold concentration as a requirement to attain a maximum killing rate is an issue with possible clinical relevance to OME, especially in the nonpurulent (serous) type of OME, where antibiotic penetration into the middle ear has been shown to be greatly reduced compared to the purulent type of OME (14).

Animal studies have reported that amoxicillin penetration into middle ear effusions is considerably less in cases of chronic (serous) versus acute (purulent) OME (14). A range of 0.2 to 4.0 mg/L of amoxicillin middle ear effusion concentrations (n = 24) following a single oral 13 mg/kg dose has been reported in children with chronic OME (7). These data, indicating amoxicillin middle ear concentrations greater than *S. pneumoniae* MIC values (16), and the *in vitro* data reported herein suggest that interpatient variability of amoxicillin middle ear penetration in patients with acute OME probably does not account for the majority of differences in patient outcome. Because these *in vitro* data are based on early single-dose kill curve studies, further investigations are required using longer experiment times (e.g., 24 hr) and/or a simulated amoxicillin multiple-dosing regimen to evaluate further this and other critical factors such as possible inoculum effects and bacterial regrowth.

The application of *in vitro* pharmacodynamic modeling techniques to the specific study of the pathogenesis of otitis media is unique. *In vitro* pharmacodynamic modeling of the various classifications of otitis media (acute, chronic, purulent, serous) has an advantage over *in vivo* modeling in that potentially confounding experimental variables can be rigidly controlled. The *in vitro* modeling of various aspects of otitis media should provide a new mechanism for further advances in understanding this disease.

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