Role of *Streptococcus pneumoniae* and *Haemophilus influenzae* in the Development of Acute Otitis Media and Otitis Media with Effusion in a Gerbil Model

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The efficacy of amoxicillin/clavulanate and cefuroxime was determined in a gerbil model of otitis media with a mixed *Streptococcus pneumoniae* plus *Haemophilus influenzae* middle ear (ME) infection. Results were compared with those obtained in a previous single *H. influenzae* model. All untreated animals inoculated with the mixed inoculum developed acute otitis media (AOM), whereas 86.7% of those inoculated with *H. influenzae* developed otitis media with effusion (OME). Antibiotics eradicated *H. influenzae* from the ME more efficiently in AOM than in OME, and this difference was highly significant (*P* < .001) after administration of 5 mg/kg of either drug (amoxicillin/clavulanate, 100% vs. 10%; cefuroxime, 73.3% vs. 10%). Efficacy was predicted by the relation of in vitro susceptibility and ME antibiotic concentration, which was 2.7 times higher in AOM than in OME. In the mixed otitis model, the most efficacious antibiotic was able to prevent AOM, but >80% of animals developed culture-negative OME.

Otitis media is the inflammation of the middle ear (ME), associated with the presence of fluid in the ME or otorrhea. *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* are the bacterial pathogens most commonly involved, with *S. pneumoniae* and *H. influenzae* being isolated in mixed culture from less than 10% up to 24% of patients [1–4]. The 2 most common conditions of otitis media are acute otitis media (AOM) and otitis media with effusion (OME). AOM usually appears with rapid onset of symptoms and marked otoscopic findings of inflammation [5]. OME is defined as an asymptomatic ME effusion that may be associated with a “plugged ear” feeling [5]. OME may be the first manifestation of an inflammatory process in the ME [5] or the evolution of a previous ear infection, which occurs in 5%–40% of AOM [6, 7]. OME has been attributed to an inflammatory process [8]. On the other hand, 40%–60% of chronic effusions are sterile by culture [9, 10], although recent studies, using polymerase chain reactions, have shown the presence of both bacterial DNA and mRNA, mostly from *Haemophilus*, even in cases of culture-negative OME [11, 12].

Treatment of otitis media, which is one of the main causes for antibiotic consumption in children, has been a matter for discussion. Although most authors favor the use of antibiotics [7, 13], there are studies showing no benefits from antibiotic therapy [14, 15]. Penicillin-insensitive *S. pneumoniae* and *H. influenzae* are increasing worldwide [16, 17]. In Spain, ME isolates show that as many as 54% of pneumococci are penicillin insensitive [18] and 23% [16] of hemophilic isolated are β-lactamase producing. This leads to the use of broad-spectrum and more expensive antibiotics.

The aims of this work have been as follows: (1) to evaluate whether the pharmacodynamic parameters in serum and the ME are predictive of bacteriological and clinical efficacies in an experimental otitis media caused by *S. pneumoniae* plus *H. influenzae*, comparing the results with those obtained in a previous single *H. influenzae* model [19]; (2) to determine whether development of OME is related to the organism involved and/or to antibiotic treatment of AOM; and (3) to compare the bacteriological and clinical responses with different antibiotics in AOM and OME. This work is part of a project for studying the course of untreated and treated AOM and OME in an animal model.

**Materials and Methods**

**Bacteria.** Two clinical isolates were used: an *S. pneumoniae* serotype 23 F (MIC for benzylpenicillin, 2 μg/mL) isolated from a patient with bacteremia and a biotype II β-lactamase-producing nonserotypeable *H. influenzae* (MIC for amoxicillin, 8 μg/mL) iso-
lated from a patient with otitis media. This strain was the same as the strain previously used in our single *H. influenzae* otitis media model [19].

**Antibiotics.** The products used for in vitro studies were lithium clavulanate and amoxicillin trihydrate (SmithKline Beecham Pharmaceuticals, Worthing, England) and cefuroxime sodium (Sigma, St. Louis). For in vivo (therapeutic) use, commercial vials of amoxicillin/clavulanate (Augmentine, 10 : 1; SmithKline Beecham Pharmaceuticals, Toledo, Spain) and cefuroxime (Curoxima; Glaxo, Madrid, Spain) were reconstituted in pyrogen free distilled water to the desired concentrations.

**In vitro studies.** MICs and MBCs were determined by microdilution methods according to National Committee for Clinical Laboratory Standards procedures [20, 21]. Median values of 5 separate determinations were considered.

**Animals.** Eight- to 9-week-old adult female Mongolian gerbils (*Meriones unguiculatus*) weighing 49 ± 5 g each were purchased from the Centre d’Élévage R. Janvier (Le Genest, St.-Ile, France). They were given unlimited access to food and water and were housed in a protected unit with slight negative pressure and a 12-h light/dark cycle. For invasive procedures, the animals were anesthetized with 50 mg/kg ketamine (Ketolar; Parke-Davis, Barcelona, Spain) and 13 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany).

**Experimental otitis.** Overnight cultures of the organisms were kept in aliquots at −70°C. For the mixed otitis media model, the day before the experiment, a freshly thawed aliquot of 400 µL of *H. influenzae* was added to 9.6 mL of brain-heart infusion broth (BHI; Oxoid, Unipath, Basingstoke, Hampshire, England) enriched with 2% Fildes extract (Oxoid) incubated at 35°C in 5% CO₂ for 20 h. On the day of the experiment, a freshly thawed aliquot of *S. pneumoniae* was incubated for 4 h at 37°C in a 5% CO₂ atmosphere in BHI enriched with 5% horse serum (Bio-Mérieux, Marcy-l’Étoile, France). Animals were inoculated bilaterally with a mixture of both broths diluted in BHI to obtain approximately with a mixture of both broths diluted in BHI to obtain 5 × 10⁵ cfu of *H. influenzae* and 5 × 10⁴ cfu of *S. pneumoniae* per 20 µL, which were introduced directly in the ME bulla. The experimental model induced by *H. influenzae* as sole bacterial pathogen was done by following previously published procedures [19]. The number of viable bacteria in the different inocula was determined by colony counting. The tympanic membrane was left intact and swelled without rupture during the inoculation. A normal tympanic aspect and correct inoculation were verified with an operating microscope. “AOM” was defined as otorrhea through a perforation in the tympanic membrane and/or inflammatory signs with changes in the membrane’s normal yellowish-pink appearance to a gray, dark brownish-yellow, or whitish opaque area, with a very rough surface texture. “OME” was defined as no inflammatory signs of tympanic membrane with air fluid levels and ME fluid with or without signs of negative ME pressure.

**Treatment regimen and efficacy studies.** Each antibiotic was tested at 2 doses (5 and 20 mg/kg), administered subcutaneously (sc) in 500 µL at 2, 10, and 18 h postinoculation. Animals in the control group received placebo (apyrogen sterile distilled water). The 5 mg/kg dose was chosen to approximate the pharmacokinetic parameters in the serum of gerbils with those obtained in humans after therapeutic oral dosing [22]. Groups of 15 animals per treatment and control groups were included. Treated and control animals were studied daily for otorrhea, weight, and behavior. Otoscopic aspect and ME samples, obtained by washing the ME fossa with 20 µL of saline solution injected and withdrawn via the epi-tympanic membrane with a 0.33-mm needle to determine bacterial counts in ME washing fluid (MEWF), were obtained on day 2 postinoculation. Aliquots of serial 10-fold dilutions in saline solution were plated on chocolate agar and sheep blood agar and incubated for 24 h at 35°C in a 5% CO₂ atmosphere. Bacterial counts are expressed as log₁₀ cfu/20 µL; the lowest detectable bacterial count was 2 cfu/20 µL (0.30 log₁₀ cfu/20 µL) for *S. pneumoniae* and 4 cfu/20 µL (0.60 log₁₀ cfu/20 µL) for *H. influenzae*. Three microliters of MEWF was extended over a 6 cm² slide surface, Gram-stained, and observed under a high-field (1000×) microscope. The mean of 10 fields was calculated and expressed as no cells (<1 per field), few (1–4 per field), moderate (5–30 per field), or many (>30 per field). Cerebrospinal fluid was obtained on day 2 by percutaneous intracysternal puncture to detect meningeal involvement.

**Pharmacokinetic studies.** Serum levels of amoxicillin/clavulanate or cefuroxime were determined in healthy animals after an sc dose of 5 or 20 mg/kg. Groups of 6 animals per dose and blood sample time (15, 30, 60, and 120 min after drug administration) were killed with CO₂ and exsanguination by intracardiac puncture. Antibiotic concentrations in ME fluid without washing (MEF) were also determined in groups of 10 animals bilaterally inoculated with *S. pneumoniae* plus *H. influenzae* or with *H. influenzae* under the same conditions as previously indicated in the experimental otitis model. Single 5 mg/kg sc doses of amoxicillin/clavulanate or cefuroxime were administered 46 h after bacterial inoculation. MEF samples were obtained 90 min later, via the epi tympanic membrane, with a 0.33-mm needle. Aliquots of MEF samples having ≥10 µL of exudate were pooled and frozen at −70°C until determination of antibiotic levels. This procedure permits collection of MEF samples at a time when effusion is consistently abundant with high bacterial density and without otorrhea.

Antibiotic concentrations were determined by microbiological assay using *Micrococcus luteus* ATCC 9341 for amoxicillin, *Bacillus subtilis* 1904E for cefuroxime, and *Klebsiella pneumoniae* NCTC 11228 for clavulanic acid. Standard curves for determination of antibiotic concentrations were obtained from standard solutions prepared in pooled gerbils’ sera for blood levels and in 0.1 mol/L phosphate buffer, pH 6.0, for MEF. Assay variability for individual samples was <10%. Pharmacokinetic analysis was performed by standard graphical methods [23].

The relationship between the maximum antibiotic serum concentration obtained and the MIC of the infecting microorganism (serum inhibitory quotient) and the relationship between the MEF antibiotic concentration after 90 min of drug administration and the MIC of the infecting microorganism (MEF inhibitory quotient, MEF level/MIC) were calculated.

**Statistical analysis.** The number of ears with a positive count over the total number of ears was calculated as a percentage in each group of animals. To detect the differences in eradication rates in each model and to compare, by group, eradication rates between the 2 models, a χ² test or Fisher’s exact test was used. To check the homogeneity of the antibiotics eradication rate between the 2 models, the Breslow-Day test was used. Bonferroni was used to adjust the global error in the comparisons of eradication rates.
Table 1. Bacterial counts expressed as arithmetic means in $\log_{10}$ cfu per 20 $\mu$L ($\pm$ SD) of middle ear washing fluid in experimental otitis media caused by Streptococcus pneumoniae plus Haemophilus influenzae (M) vs. H. influenzae alone (S).

<table>
<thead>
<tr>
<th>Group (dose, mg/kg)</th>
<th>S. pneumoniae$^a$</th>
<th>H. influenzae$^b$</th>
<th>H. influenzae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>3.3 ± 1.0</td>
<td>4.0 ± 1.6</td>
<td>5.0 ± 1.3</td>
</tr>
<tr>
<td>Amoxicillin/clavulanate (5)</td>
<td>0.3 ± 0.1</td>
<td>0.6 ± 0.0$^c$</td>
<td>4.9 ± 1.8$^e$</td>
</tr>
<tr>
<td>Amoxicillin/clavulanate (20)</td>
<td>0.3 ± 0.0</td>
<td>0.6 ± 0.0</td>
<td>0.8 ± 1.2$^c$</td>
</tr>
<tr>
<td>Cefuroxime (5) $^f$</td>
<td>4.7 ± 1.7$^e$</td>
<td>1.5 ± 1.7$^e$</td>
<td>4.7 ± 1.7$^e$</td>
</tr>
<tr>
<td>Cefuroxime (20)</td>
<td>1.2 ± 1.3</td>
<td>0.6 ± 0.0$^c$</td>
<td>3.9 ± 2.7$^e$</td>
</tr>
</tbody>
</table>

NOTE. Data are mean ± SD.

a Limit of detection $\log_{10}$ 0.3 (2 cfu/20 $\mu$L).
b Limit of detection $\log_{10}$ 0.6 (4 cfu/20 $\mu$L).
c Significant differences ($P \leq .001$) by dose and antibiotic, comparing colony counting in the mixed versus the single model.

Otorrhea was analyzed by use of a $\chi^2$ test. Bacterial counts were expressed in untreated and treated animals as arithmetic means in $\log_{10}$ cfu per 20 $\mu$L of MEWF, culture-negative samples being included in the calculation of means assuming a value at the detection limit. Analysis of covariance (ANCOVA) was used to compare $\log_{10}$ cfu, MEWF recovered, and weight values in each antibiotic group controlled for the $\log_{10}$ cfu or weight basal values and for the differences by antibiotic group between the 2 models. Analysis of variance (ANOVA) was used to compare MEWF volume. When the ANCOVA or ANOVA $P$ value was significant ($P < .05$), contrast between groups was made by use of the Tukey-Kramer test to adjust the type I experiment-wise error.

**Results**

In vitro studies. For H. influenzae, median MICs/MBCs were 1/1 $\mu$g/mL for both amoxicillin/clavulanate and cefuroxime and 1/1 and 4/4 $\mu$g/mL, respectively, for S. pneumoniae.

Therapeutic efficacy in experimental otitis. Figure 1 shows the otoscopic and bacteriological results at day 2 in both models in relation to the percentage of positive samples for either S. pneumoniae or H. influenzae or both. After inoculation of the mixed inoculum, bilateral AOM was obtained in all untreated animals at day 2 with 100% of culture-positive MEWF samples, 80% with both organisms, and 20% with a single organism recovered (16.7%) with S. pneumoniae and 3.3% with H. influenzae. Most MEWF specimens contained moderate amounts of polymorphonuclear cells with intra- and extracellular organisms. Animals were hypoactive, with otorrhea and a significant weight loss. After the single inoculation of H. influenzae, unilateral or bilateral otitis media was obtained in all untreated animals at day 2, with 86.7% of the animals showing OME and only 13.3% having AOM, with a total of 96.7% of MEWF specimens being culture positive. Most MEWF specimens contained a moderate number of polymorphonuclear cells with intra- and extracellular hemophili. Animals remained active, without otorrhea and with no significant weight loss.

In the mixed model, animals treated with amoxicillin/clavulanate did not show AOM, although $\geq$83% developed a post-treatment OME. Approximately 60% and 30% of animals treated with cefuroxime had AOM and OME, respectively. The presence of AOM was related to the persistence of S. pneumoniae in both untreated and treated animals. In the H. influenzae model, all animals, untreated or treated with either antibiotic and either dose, showed OME in $\geq$70% of the cases, with no relation to the number of culture-positive specimens.

The bacteriological outcome in the mixed model showed a statistically significant difference ($P \leq .001$) when comparing the eradication percentage in each treatment group versus control. Amoxicillin/clavulanate at any dose was able to achieve $\geq 90\%$ (93.3%–100%) of culture-negative MEWF specimens, whereas cefuroxime achieved between 20% (5 mg/kg) and 53.3% (20 mg/kg). The poor results obtained with cefuroxime were due to the persistence of S. pneumoniae (80% and 46.7% with the low and high dose, respectively). Considering only S. pneumoniae, amoxicillin/clavulanate at any dose obtained an eradication rate significantly ($P \leq .001$) higher than the rate obtained in any of the cefuroxime or control groups; cefuroxime showed significant differences ($P \leq .001$) from control only at the high dose. With respect to H. influenzae, the high dose of cefuroxime achieved a 100% eradication, but after the low dose, 26.7% of MEWF samples yielded growth of both H. influenzae and S. pneumoniae. The comparison of bacteriological results in both models in relation to the H. influenzae outcome shows that only the high dose of amoxicillin/clavulanate obtained eradication rates $\geq 90\%$ in both models. Significant differences ($P \leq .001$) in eradication rates between the mixed and single models were found for all groups, except for the control group and the high-dose amoxicillin/clavulanate group. H. influenzae was more efficiently eradicated in the mixed model than in the single model, making eradication rates in any treatment group statistically significantly ($P \leq .001$) higher than those in the control group, whereas in the single model only the high dose of cefuroxime or amoxicillin/clavulanate demonstrated differences ($P < .0013$) with control. No animals, treated or untreated, in the mixed or the single model developed meningitis.

Table 1 presents the results of bacterial counts obtained in both the mixed and the single models of otitis media. When treatment group H. influenzae counts in the mixed versus the single model were compared, significant differences ($P \leq .001$) were found for all treatment groups except for the control and the high-dose amoxicillin/clavulanate group. Considering only the mixed model, amoxicillin/clavulanate at any dose and the high dose of cefuroxime were able to reduce significantly ($P \leq .001$) the number of both S. pneumoniae and H. influenzae, compared with untreated animals, whereas cefuroxime at low dose reduced significantly ($P \leq .001$) only the number of hemophili. In the H. influenzae model, only the high dose of amoxicillin/clavulanate was able to reduce significantly ($P = .00004$) the number of organisms, compared with any other group.
Again, *H. influenzae* was more efficiently eradicated in the mixed model than in the single one.

Table 2 presents the relationship among otoscopic examination, presence of otorrhea, volume recovered after 20 μL saline washing of ME, and variation in body weight in the mixed model. The detection of AOM or OME was related to the presence/absence of otorrhea and the volume of MEWF recovered: the highest volumes were found in the groups of animals with the lowest percentages of otorrhea and AOM, but with OME (those treated with any dose of amoxicillin/clavulanate), the lowest volumes were related to the highest percentages of AOM and otorrhea (untreated animals and those receiving any dose of cefuroxime). In the *H. influenzae* otitis model (data not shown), most cases were OME, without differences in the volume recovered from any group (treated or untreated) of animals. Untreated animals had a higher mean volume of MEWF than that obtained in the mixed model (34.3 ± 9.8 vs. 23.3 ± 6.4 μL).

The greatest body weight loss occurred in untreated animals, followed by those treated with any dose of cefuroxime, in relation to presence of AOM. Nevertheless, statistically significant differences (*P* < .001) were found only between control or cefuroxime low-dose and any other group. The lowest body weight loss was observed in animals receiving the low dose of amoxicillin/clavulanate, where no AOM cases were detected. However, although no case of AOM was observed in animals treated with the high dose of amoxicillin/clavulanate, the body weight loss in this group was similar to that observed in the cefuroxime high-dose group. In the *H. influenzae* model, animals showed little variation in their body weight, with a good relation to low rate of AOM. A significant reduction in the body weight was observed with the high dose of amoxicillin/clavulanate when compared with those in any of the other groups (*P* < .05).

**Pharmacokinetic and pharmacodynamic data.** Of the 4 serum samples obtained for the pharmacokinetic study, the sample taken 15 min after drug administration showed the highest concentration (C₁₅ₐₘᵢₜ) for any antibiotic and dose and was
Table 2. Relationship between otoscopic examination, presence of otorrhea, volume (µL) of middle ear washing fluid recovered, and variation in body weight in an experimental otitis media caused by *Streptococcus pneumoniae* plus *Haemophilus influenzae*.

<table>
<thead>
<tr>
<th>Group (dose, mg/kg)</th>
<th>AOM (%)</th>
<th>OME (%)</th>
<th>Normal (%)</th>
<th>Otorrhea, µL</th>
<th>Volume, mean ± SD</th>
<th>% Body weight loss, mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control (0)</td>
<td>100.0</td>
<td>0.0</td>
<td>0.0</td>
<td>100.0</td>
<td>23.3 ± 6.4</td>
<td>–11.1 ± 1.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Amoxicillin/clavulanate (5): 38.4 ± 14.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.0</td>
<td>93.3</td>
<td>6.7</td>
<td>10.0</td>
<td>38.4 ± 4.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>–1.1 ± 2.6</td>
</tr>
<tr>
<td>Cefuroxime (5)</td>
<td>58.6</td>
<td>26.7</td>
<td>6.7</td>
<td>90.0</td>
<td>27.7 ± 8.8</td>
<td>–8.8 ± 3.9&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cefuroxime (20)</td>
<td>58.6</td>
<td>37.9</td>
<td>3.4</td>
<td>86.7</td>
<td>29.3 ± 12.1</td>
<td>–4.5 ± 3.4</td>
</tr>
</tbody>
</table>

**NOTE.** Amoxicillin, amoxicillin/clavulanate; AOM, acute otitis media; OME, otitis media with effusion.

<sup>a</sup> Thirty otoscopic examinations were performed for all groups except cefuroxime (20), where only 29 examinations were valuable. Data are percentages.

<sup>b</sup> Chi-square test, 92.37 (<i>P</i> < .001).

<sup>c</sup> Significant differences (<i>P</i> < .001) with any other group.

<sup>d</sup> Significant differences (<i>P</i> < .05) with control and low dose of cefuroxime.

Table 3. Serum pharmacodynamic data of amoxicillin/clavulanate and cefuroxime in relation to eradication of organisms in *Streptococcus pneumoniae* plus *Haemophilus influenzae* (M) and *H. influenzae* alone (S) models.

<table>
<thead>
<tr>
<th>Group (dose, mg/kg)</th>
<th>C&lt;sub&gt;15 min&lt;/sub&gt;/MIC</th>
<th>t &gt; MIC, min</th>
<th>Eradication, %</th>
<th>C&lt;sub&gt;15 min&lt;/sub&gt;/MIC</th>
<th>t &gt; MIC, min</th>
<th>Eradication, %</th>
<th>C&lt;sub&gt;15 min&lt;/sub&gt;/MIC</th>
<th>t &gt; MIC, min</th>
<th>Eradication, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin/clavulanate (5)</td>
<td>10.1</td>
<td>109.0</td>
<td>93.3</td>
<td>10.1</td>
<td>109.0</td>
<td>100.0</td>
<td>10.1</td>
<td>109.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Amoxicillin/clavulanate (20)</td>
<td>40.8</td>
<td>119.0</td>
<td>100.0</td>
<td>40.8</td>
<td>119.0</td>
<td>100.0</td>
<td>40.8</td>
<td>119.0</td>
<td>91.7</td>
</tr>
<tr>
<td>Cefuroxime (5)</td>
<td>2.5</td>
<td>36.0</td>
<td>20.0</td>
<td>10.1</td>
<td>60.0</td>
<td>73.3</td>
<td>10.1</td>
<td>60.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Cefuroxime (20)</td>
<td>5.7</td>
<td>55.0</td>
<td>53.3</td>
<td>23.3</td>
<td>105.0</td>
<td>100.0</td>
<td>23.3</td>
<td>105.0</td>
<td>36.7</td>
</tr>
</tbody>
</table>

**NOTE.** Amoxicillin, amoxicillin/clavulanate.

Discussion

AOM is related to infection by organisms with high pathogenic potential, such as *S. pneumoniae*, in humans [16, 24, 25] and in experimental models [26, 27]. In contrast, OME seems to be related to infection by less pathogenic organisms, such as nonserotypeable *H. influenzae* strains, mainly from biotype II, as has been confirmed in humans [24, 28] and experimental models [19].

Animal models of otitis media caused by *S. pneumoniae* or *H. influenzae* have been described, but otitis media due to mixed infection (*S. pneumoniae* plus *H. influenzae*) has not been previously reported. Although this mixed etiology is not common in humans, the model allowed us to better understand the pharmacodynamic principles and bacterial cooperation. In this experimental model, all animals challenged with a mixed *S. pneumoniae*/*H. influenzae* inoculum developed AOM, whereas challenge with *H. influenzae* culture alone caused OME in 86.7% of the animals. In an earlier study [26], we obtained AOM after inoculating a pure culture of the same *S. pneumoniae* strain used in the present work. This confirms that both conditions (AOM and OME) may depend on the organism involved. It should also be noted that the development of AOM or OME in a mixed model depends on the relative amount of *S. pneumoniae*/*H. influenzae* in the inoculum: *S. pneumoniae* predominance induces AOM, and *H. influenzae* predominance induces OME (present authors, unpublished data).

Another factor to be taken into account is the time from inoculation to diagnosis. In the previous model of otitis media by *S. pneumoniae*, all untreated animals developed AOM 2 days
after bacterial inoculation, but, 24 h later, as many as 32% of these animals developed OME (present authors, unpublished data), suggesting that the latter is an intermediate step between AOM and recovery.

The results obtained in the different treatment groups in this mixed model also suggest that bacteriological success in AOM leads to the appearance of OME, because, despite the high efficacy of amoxicillin/clavulanate in AOM treatment, a high rate of posttreatment OME was observed. This could be related to the inflammatory process induced by antimicrobial killing of pneumococci, as has been demonstrated in an experimental otitis media model in chinchillas [29]. In the clinical practice, the natural history of appropriately treated AOM seems also to include persistent ME effusions for several weeks in the majority of children [7, 30].

AOM or OME also affects the gerbils' behavior and weight loss patterns, with AOM leading to a greater decrease in activity and weight than was the case in OME. We did not perform blood cultures to the animals, so we do not know if such weight loss was related to systemic involvement, although central nervous system infection was ruled out by CSF cultures. In humans, S. pneumoniae/AOM presence also implies more severe clinical symptoms/signs [25, 31]. In general, the loss of weight in treated animals correlates with AOM treatment failure, but a higher weight loss than the one expected was observed with 20 mg/kg of amoxicillin/clavulanate in both models.

The AOM produced in this mixed model was effectively treated with 3 amoxicillin/clavulanate doses, both from the bacteriological and clinical (animals more active without otorrhea and with none or very little ponderal loss) point of view. On the contrary, cefuroxime, even at the high dose, failed in almost 50% of the animals due to S. pneumoniae persistence. The efficacy of antibiotics was related to the antibiotic dose and the MIC of the organism involved. Although the S. pneumoniae strain used showed an intermediate susceptibility to amoxicillin [20], 5 mg/kg of amoxicillin/clavulanate was able to eradicate the pathogen due to the favorable pharmacokinetic and pharmacodynamic parameters obtained in both serum and MEF. The poorer results with cefuroxime can be attributed to the inadequate serum and MEF pharmacodynamic parameters, because of the resistant categorization of the strain [20]. The H. influenzae strain used can be considered fully susceptible to both amoxicillin/clavulanate and cefuroxime [20], and eradication rates >70% in the mixed model were obtained with both antibiotics. This was not the case in the single H. influenzae model, where amoxicillin/clavulanate at high dose was the only therapeutic regimen that obtained efficacy (eradication rate >90%).

Particularly important is the fact that the antibiotics were much more efficacious in eradicating H. influenzae in the model of mixed infection (i.e., in AOM) than in the single H. influenzae infection (i.e., in OME), due to the higher MEF antibiotic concentration in the mixed model, probably related to the higher inflammatory response observed in AOM than in OME. This confirms that results are predicted by MEF pharmacodynamic data but not by serum data.

Several experimental facts support that in AOM, antimicrobial treatment should be promptly established (when drug diffusion is favored by the strong inflammatory process), perhaps in short courses. As inflammation diminishes with time and with antibiotic treatment, the drug may not achieve a sufficiently high concentration in the MEF, thus decreasing its effectiveness, which agrees with the clinical evidence that 2, 3, 5, and 10 days of treatment are equally effective [32, 33]. Poorer penetration of penicillin into the ME after the second day of treatment was already reported 20 years ago [34]. In OME, where inflammatory response is lower, antibiotics have demonstrated less efficacy, as described in several reports showing bacteriological failures in children treated for otitis media caused by H. influenzae [28]. For this reason, some authors do not recommend the use of antibiotics in OME, except under specific circumstances [35]. In the chinchilla model, it has also been demonstrated that antibiotic penetration across the ME membrane is strongly influenced by the degree of ME inflammation [36].

In summary, the comparisons of the mixed versus H. influenzae model have shown the following: (1) the appearance of AOM or OME may depend, among other factors, on the type of organism involved, the time since the appearance of the first symptoms to diagnose, and whether a previous antibiotic has been administered; (2) the poorer eradication rates with lower inflammation (i.e., H. influenzae, OME); (3) the increase of inflammation by the presence of S. pneumoniae, which induces a higher MEF inhibitory quotient and higher rate of bacterial eradication (i.e., H. influenzae, AOM); and (4) the better predictive value of MEF than of serum pharmacodynamic parameters.

### Table 4. Middle ear fluid (MEF) pharmacodynamic data of amoxicillin/clavulanate and cefuroxime in relation to eradication of organism in Streptococcus pneumoniae plus Haemophilus influenzae (M) and H. influenzae alone (S) models.

<table>
<thead>
<tr>
<th>Group (dose, mg/kg)</th>
<th>MEF level/MIC</th>
<th>Eradication, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pneumoniae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxi/clav (5)</td>
<td>2.4</td>
<td>93.3</td>
</tr>
<tr>
<td>Cefuroxime (5)</td>
<td>0.5</td>
<td>20.0</td>
</tr>
<tr>
<td>H. influenzae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxi/clav (5)</td>
<td>2.4</td>
<td>100.0</td>
</tr>
<tr>
<td>Cefuroxime (5)</td>
<td>2.2</td>
<td>73.3</td>
</tr>
<tr>
<td>H. influenzae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amoxi/clav (5)</td>
<td>0.9</td>
<td>10.0</td>
</tr>
<tr>
<td>Cefuroxime (5)</td>
<td>0.8</td>
<td>10.0</td>
</tr>
</tbody>
</table>

a Antibiotic level in MEF 90 min after drug administration. Amoxi/clav, amoxicillin/clavulanate.
Acknowledgments

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References