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Effect of Increased Dosages of Amoxicillin in Treatment of Experimental Middle Ear Otitis Due to Penicillin-Resistant *Streptococcus pneumoniae*

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A gerbil model of acute middle ear otitis was used to evaluate the efficacy of increased dosages of amoxicillin in eradicating infection induced by penicillin-resistant *Streptococcus pneumoniae*. Three different strains were used: (i) a serotype 23 penicillin-susceptible strain; (ii) a serotype 23 penicillin-resistant strain (MIC of penicillin, 2 μg/ml); and (iii) a serotype 19 highly penicillin-resistant strain (MIC of penicillin, 4 to 8 μg/ml). Animals were inoculated bilaterally with 10^7 CFU per ear by transbulla challenge and treated 2 to 4 h postinfection by amoxicillin administrated subcutaneously. The course of the disease was monitored bacteriologically on days 2, 4, and 8 postinfection. The three strains had a similar pathogenicity in untreated animals in terms of the duration of the disease, bacterial counts in middle ear (ME) fluid, and systemic complications. Infection due to the penicillin-susceptible strain was cured after two injections of 2.5 mg/kg of body weight. No bacteria were recovered at day 2 after two injections at 10 and 25 mg/kg with the penicillin-resistant and highly penicillin-resistant strains, respectively. Under these experimental conditions, increased doses of amoxicillin consistent with MICs were able to clear ME infection. Pharmacokinetic parameters of amoxicillin in serum and ME fluid were within the clinical range at the doses used in the study.

Emergence of *Streptococcus pneumoniae* strains with decreased susceptibility to penicillin is a worldwide problem. Pharyngeal carriage, frequent exposure to antibiotics, and low immunity against noninvasive strains explain the high isolation rate of these strains in children (11). Epidemiological studies show an increasing incidence of penicillin-resistant strains of *S. pneumoniae* (SpRP) in most countries. This involved 70% of *S. pneumoniae* strains isolated in Hungary from ear, nose, throat, and sinus swabs in 1988 to 1989 (13), 33% of *S. pneumoniae* isolated from middle ear (ME) fluid cultures in a children's hospital in the United States in 1989 (3), and 22% of isolates in France in 1990 (6). Empiric treatment of acute otitis media must take these strains into account but is limited by the frequent resistance of *S. pneumoniae* to other oral antimicrobial agents.

Group A penicillins remain the most microbiologically effective oral beta-lactam antibiotic (6, 14), and increased standard clinical dosages are thought to be sufficient to treat otitis media due to *S. pneumoniae* with decreased susceptibility to penicillin. Recently, this solution was proposed during the consensus conference on respiratory tract infections of the Société de Pathologie Infectieuse de Langue Française (16).

To evaluate experimentally the efficacy of this approach, we studied the response of SpRP ME infection to various dosages of amoxicillin in gerbils. Three different *S. pneumoniae* strains with similar local and meningeal pathogenicity were used as the infecting agents: a serotype 23 strain with markedly decreased susceptibility to penicillin (MIC, 2 μg/ml), a serotype 19 highly resistant strain (MIC, 4 to 8 μg/ml), and a serotype 23 susceptible strain (SpSP) as reference. Pharmacokinetic studies of amoxicillin in gerbil serum and ME effusions were also performed.

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MATERIALS AND METHODS

This study was performed in accordance with prevailing regulations regarding care and use of laboratory animals in the European Communities (Journal officiel des Communautés Européennes, 18 December 1986, L358).

Animals. Eight- to 9-week-old adult female Mongolian gerbils (body weight range, 40 to 50 g) were purchased at the Centre d'Elevage R. Janvier (Le Genest Saint Isle, France). Animals were given free access to food and water and housed in a protected unit with slight negative pressure, filtered air, and a 12-h light–12-h dark cycle (Iffa Credo, l'Arbresle, France). For all invasive procedures, the animals were anesthetized by intramuscular injection of a mixture of 40 mg of ketamine (Ketalar; Parke-Davis, Courbevoie, France) per kg of body weight and 13 mg of xylazine (Rompun; Bayer Pharma, Sens, France) per kg. One week prior to the experiments, the antero-inferior part of the auricle was removed to facilitate access to the eardrum.

Infecting organisms. The three differents strains of *S. pneumoniae* are identified in the text (Table 1) as SpSP, serotype 23 strain GA01 isolated from human blood and provided by E. Vallée (Hôpital Bichat-Claude Bernard, Paris, France); SpRP-1, serotype 23 strain 54B isolated from acute sinusitis in an adult and provided by I. Boucot (Hôpital Necker-Enfants Malades, Paris, France); and SpRP-2, serotype 19 strain 15986 isolated from a patient with acute otitis media and provided by P. Geslin (Centre National de Référence du Pneumocoque, Créteil, France). SpRP-1 and -2 are also resistant to other classes of antibiotics, in particular, macrolides and sulfamides.

TABLE 1. Microbiologic data for the study strains[a]

Strain	Penicillin		Amoxicillin	
	MIC	MBC	MIC	MBC
SpSP (GA 01)	0.016	0.03	0.016	0.016
SpRP-1 (54 B)	2	2–4	1	2
SpRP-2 (15986)	4–8	8	2–4	4

[a] Values are given in micrograms per milliliter.

The MICs and MBCs were determined in Mueller-Hinton infusion broth (Diagnostic Pasteur) by the tube dilution method (National Committee for Clinical Laboratory Standards). Tubes containing serial twofold dilutions of antibiotic and a final bacterial density of 10^6 CFU/ml were incubated for 18 h at 37°C with 10% CO_2-air. The MIC was defined as the lowest concentration of antibiotic at which no growth was visible to the naked eye. For the determination of the MBC, 0.01-ml aliquots from tubes with no visible growth were plated onto Columbia agar with 5% sheep blood (Bio-Merieux) and incubated overnight at 37°C in 10% CO_2-air. The MBC was defined as the lowest concentration killing >99.9% of the original inoculum.

Virulence was maintained by passage in mice. Aliquots of pneumococcal suspensions were stored at −80°C. On each day of experimentation, a freshly thawed aliquot was incubated for 6 h at 37°C in brain-heart infusion broth (Bio-Merieux, Lyon, France) enriched with 5% horse serum. This culture was suspended in 0.9% saline to the desired concentration. The number of viable bacteria in the inoculum was determined by the pour-plating colony count method.

Experimental otitis. Animals were inoculated bilaterally with 20 μl of bacterial suspension introduced directly in the ME bulla according to the procedure originally described by Giebink et al. for the chinchilla (9). 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. The minimal pathogenic inoculum was 10^4 CFU per ear. An increased inoculum (10^6 to 10^7 CFU per ear) was required to induce acute ME otitis and to evaluate amoxicillin treatment.

Treatment. Amoxicillin sodium salt (Clamoxyl; Beecham, Paris, France) was reconstituted according to the instructions in the package insert and diluted in sterile water to the desired concentration. Treatment was initiated 2 to 4 h postinfection (p.i.) and administrated subcutaneously (s.c.) b.i.d. or t.i.d. in 500 μl. Dosage regimens are given in Results. In all procedures, a control group was injected s.c. with sterile water under identical conditions.

Follow-up. Treated and control animals were studied longitudinally for weight, behavior, otoscopic aspect, and bacterial counts in ME effusion. To detect meningeal involvement, bacterial counts in cerebrospinal fluid (CSF) were also determined. ME samples were obtained by washing the ME fossa with 20 μl of saline injected and withdrawn via the epitympanic membrane with a 0.3-mm needle. Complete healing of the puncture occurred within 2 days, and the washing procedure did not alter the course of the disease (personal data). CSF was obtained by percutaneous intracysternal puncture. Sampling of a mean 30-μl volume was well tolerated.

In the control groups and groups treated for 1 day, bacterial counts were determined at days 2, 4, and 8 p.i. in ME washings and at days 2 and 4 in CSF samples. In groups treated for 2 days, bacterial counts in ME washings and in CSF were determined at day 4 p.i.; bacterial regrowth in ME fluid was evaluated by further sampling 3 days later.

Bacterial counts. Shortly after sampling, 100-μl aliquots of serial 10-fold dilutions in saline were plated on sheep blood-agar. Plates were incubated for 18 h at 37°C. Bacterial counts are expressed as $\log_{10}$ CFU/20 μl of ear washing fluid.

Pharmacokinetic studies. Pharmacokinetic parameters of amoxicillin were determined in sera and ME effusions of animals inoculated with 40 CFU of *S. pneumoniae* serotype 3 per ear. At 3 days p.i., animals received a single s.c. dose of 100 mg/kg and were sacrified at 0.25, 0.5, 1, 2, 4, and 6 h after drug administration. At each time, serum samples and ME washings were collected from three animals.

The relationship between the dose and peak antibiotic level in serum was determined for uninfected animals. Groups of five animals were injected s.c. with 10, 25, 50, or 100 mg/kg, and serum samples were collected 0.5 h later.

Amoxicillin concentrations were measured by the agar disk diffusion method with *Sarcina lutea* as the test strain. The detection limit of the assay was approximately 0.06 μg/ml. Amoxicillin was assayed in pure serum and in five-fold-diluted ME washings. Antibiotic levels in the serum and ME fluid of each animal were calculated from duplicate or triplicate assays. Mean values were determined for each time-dose group.

Expression of the results and statistical analysis. The lowest detectable bacterial count in ME washing fluid was 0.34 $\log_{10}$ CFU/20 μl. ME effusion was considered sterile when bacterial counts in washing fluid were below the detection limit. The percentage of acute ME otitis is the number of animals with a positive count in at least one ear over the total number of animals in the treatment group. This percentage recorded in each amoxicillin-treated group was compared with that of the untreated control at the same day p.i. by Yates' χ^2 test with continuity correction. Mean bacterial counts (both ears) for each treatment group are expressed as $\log_{10}$ CFU ± standard error for n representative animals. Mean counts at the same day p.i. were compared between treatment groups by variance analysis, and Fisher's test was applied to compare the results of each amoxicillin regimen with that of the untreated control. In CSF, the lowest detectable bacterial count ranged from 1.8 to 2 $\log_{10}$ CFU/ml, and counts below the detection limit were considered negative. In the pharmacokinetic study, linear regression was applied to compute the elimination half-life in serum and ME effusion; correlations between the maximal concentration of amoxicillin in serum and the dose administrated were sought by using the linear regression coefficient r. P values of ≤0.05 were considered significant.

RESULTS

Infective dose. Animals inoculated with 10^4 CFU per ear of the SpRP-1 strain were studied from days 1 to 8 p.i. The body weight curve was not altered by infection. Only slight tympanic inflammation was visible, but effusion was always present. Bacterial counts were below the limit of detection in all animals after day 1 p.i., but ME inflammation was shown by an increased number of inflammatory cells in ME washing, which reached 10^5/20 μl (85% polymorphonuclear leukocytes) at day 3 p.i. Similar results were obtained with a 10^4-CFU inoculum of the SpSP strain: slight tympanic inflammation and short-lived self-limiting infection. No infective dose study was performed with the SpRP-2 strain.

With an inoculum of 10^7 CFU per ear, purulent otitis media was obtained in 90 to 100% of the animals with the

TABLE 2. Outcome of ME infection induced by three *S. pneumoniae* strains with various amoxicillin regimens

Strain and amoxicillin dosage (mg/kg)[a]	Treatment duration	Bacterial status of ME effusion at day p.i.									Animals with culture-positive CSF			
		2			4			8			On days 2–4		Survivors	
		Acute ME otitis[b]		Mean counts[c]	Acute ME otitis		Mean counts	Acute ME otitis		Mean counts				
		No.	%		No.	%		No.	%		No.	%	No.	%
SpSP														
None		9/10	90	2.3 ± 0.4	9/10	90	2.7 ± 0.5	6/9	67	1.6 ± 0.5	5	50	4	80
2.5 b.i.d.	1 day	0/12[d]	0	<0.34[d]	1/11[e]	9	0.6 ± 0.2[d]	1/10[f]	10	0.39 ± 0.05[f]	2	17	2	100
SpRP-1														
None		15/15	100	2.7 ± 0.3	10/13	77	2.7 ± 0.5	7/13	54	1.2 ± 0.4	5	33	3	60
2.5 b.i.d.	1 day	3/6[f]	50	1.2 ± 0.6[d]	4/4	100	1.9 ± 0.6	1/4	25	0.5 ± 0.2	6	100	4	67
10 b.i.d.	1 day	0/11[d]	0	<0.34[d]	9/11	82	2.5 ± 0.4	3/11	27	0.6 ± 0.2	0	0		
10 b.i.d.	2 days				8/10	80	2.1 ± 0.4	5/10	50	1.2 ± 0.4	1	10	1	100
10 t.i.d.	1 day	2/7[e]	29	0.5 ± 0.1[d]	5/7	71	1.2 ± 0.4[f]	0/6	0	<0.34[f]	0	0		
10 t.i.d.	2 days				3/8	38	0.9 ± 0.3[e]	0/8[f]	0	<0.34[f]	1	13	1	100
25 b.i.d.	1 day	0/10[d]	0	<0.34[d]	4/10	40	0.9 ± 0.3[e]	1/9	11	0.41 ± 0.07[f]	3	30	3	100
SpRP-2														
None		11/11	100	2.8 ± 0.4	8/11	73	2.1 ± 0.7	4/10	40	1.2 ± 0.4	6	55	3	50
10 b.i.d.	1 day	4/7	57	2.3 ± 1.0	4/6	67	2.0 ± 0.8	1/4	25	0.5 ± 0.1	4	57	1	25
10 b.i.d.	2 days				2/5	40	0.8 ± 0.4	0/2	0	<0.34	2	40	0	0
25 b.i.d.	1 day	1/12[d]	8	0.35 ± 0.01[d]	0/12[e]	0	<0.34[e]	1/12	8	0.43 ± 0.09	2	17	2	100

[a] For each strain, animals were inoculated bilaterally by transbulla challenge with 10^7 CFU per ear.
[b] Determined by culture-positive ME washings.
[c] Mean $\log_{10}$ CFU ± standard error in 20 μl of washing fluid for the total number of animals in the group. The detection limit was 2.2 bacteria (0.34 $\log_{10}$ CFU).
[d] $P < 0.001$ versus untreated controls on the same day p.i.
[e] $P < 0.01$ versus untreated controls on the same day p.i.
[f] $P < 0.05$ versus untreated controls on the same day p.i.

three strains studied, and the course of the disease was similar (Table 2). At day 2 p.i., animals showed a significant (10.4% ± 0.4%, $n = 36$) weight loss compared with their preinoculation weights. At day 2, otoscopic examination showed marked inflammation with retrotympanic exsudate and frequent bulging of the tympanic membrane. Spontaneous bacterial count reductions were observed after day 4 p.i. Complete eradication of infection was achieved within 8 to 15 days in most animals: at day 8, 15 of 32 (47%) animals had sterile ME effusion. Meningeal complications (bacteria isolated in CSF) occurred in 16 of 36 (44%) animals but were not always lethal, as 10 (60%) animals survived.

Antibiotic assay. The response to amoxicillin treatment of ME infection induced by the penicillin-susceptible strain is shown in Table 2. With 1-day 2.5-mg/kg b.i.d. treatment, bacterial counts in ME washings were under the detection limit at day 2 p.i. in all animals. Bacterial regrowth at day 4 was observed in only 1 of 11 treated animals. The presence of bacteria in CSF at day 2 or 4 p.i. was less frequent in treated animals than in controls, but no statistical difference was found.

The response to amoxicillin treatment of ME infection induced by the SpRP-1 strain is shown in Table 2. One-day treatment at 2.5 mg/kg b.i.d. failed to clear the ME infection at 2 days p.i. in three of six animals. Two animals with no detectable bacteria in ME washings but positive CSF cultures died. All surviving animals had detectable bacteria in ME washings at day 4. With 1-day treatment at 5 mg/kg b.i.d., ME infection was observed in 4 of 10 animals at day 2, and bacterial regrowth was detected at day 4. Two-day treatment with the same regimen did not prevent bacterial persistence at day 4 (five of five animals). After 1-day 10-mg/kg b.i.d. treatment, no bacteria were recovered at day 2, but bacterial regrowth at day 4 was frequent with both 1- and 2-day treatments (in 9 of 11 and 8 of 10 animals, respectively). A t.i.d. regimen for 1 or 2 days resulted in decreased mean bacterial counts at day 4 and negative ME washings at days 7 to 8. One-day treatment at 25 mg/kg b.i.d. had similar effects but did not completely prevent bacterial regrowth at day 4. Microbiologic assays performed on the bacteria cultured after regrowth did not show increased resistance to amoxicillin. The rate of CSF involvement was significantly reduced in groups treated with 10- or 25-mg/kg regimens ($P < 0.05$). Amoxicillin treatment at 50 mg/kg b.i.d. was highly toxic (abdominal disorders and marked body weight loss); three of six animals died.

The results obtained with the highly penicillin-resistant strain are presented in Table 2. One- or 2-day treatment at 10 mg/kg b.i.d. was not sufficient to clear ME infection, but a complete cure was obtained with 1-day treatment at 25 mg/kg b.i.d. A few bacteria were recovered at day 2 in ME washings for 1 of the 12 treated animals, but ME effusions were sterile at days 4 and 8 p.i. Positive CSF cultures at days 2 and 4 were less frequent in the group treated with the highest daily dose but not significantly so.

Pharmacokinetic data. In infected animals, amoxicillin concentrations in serum reached a maximum of 53 ± 7 μg/ml at 0.5 h after a single s.c. injection of 100 mg/kg (Fig. 1); the serum elimination half-life was 0.8 h. The mean concentration in ME washings was maximal and constant 1 to 2 h after injection (6.8 ± 1.6 μg/ml); the elimination half-life was 1.6 h.

Amoxicillin was measured in washing fluid, which is about 2.5-fold diluted relative to pure ME effusion (personal data); this correction factor was applied to concentrations of amoxicillin in pure ME effusion represented in Fig. 1. In these conditions, the ME/serum ratio of the maximal amoxicillin concentration was 32%.

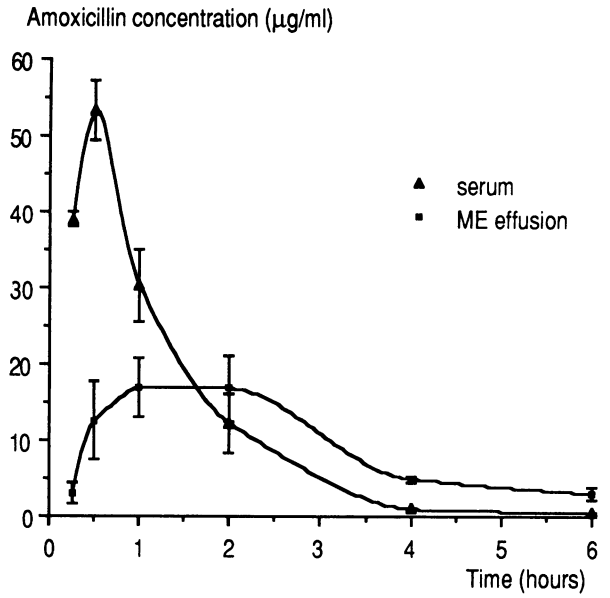

FIG. 1. Concentration-time curves of amoxicillin in serum and ME effusion of gerbils following a single s.c. dose of 100 mg/kg. Animals were inoculated by transbulla pneumococcal challenge 3 days before the assay. Values are representative means ± standard errors for three animals.

After a single s.c. injection of 100 mg/kg, mean peak levels in serum were similar in infected and uninfected animals. Peak levels in serum were dose dependent ($P < 0.01$) at the dosages studied, with no direct proportionality (Fig. 2).

DISCUSSION

The experimental model of acute ME otitis originally described by Giebink et al. (8) for chinchillas was adapted to gerbils by Fulghum et al. (4) and recently used in the latter species to evaluate antimicrobial efficacy (2, 10, 17).

S. pneumoniae strains with decreased susceptibility to penicillin belong to the noninvasive serotypes (mainly serotypes 6, 19, and 23) (11). Animals species generally used for experimental models of ME otitis (chinchillas and gerbils) present various susceptibilities to infection by these strains

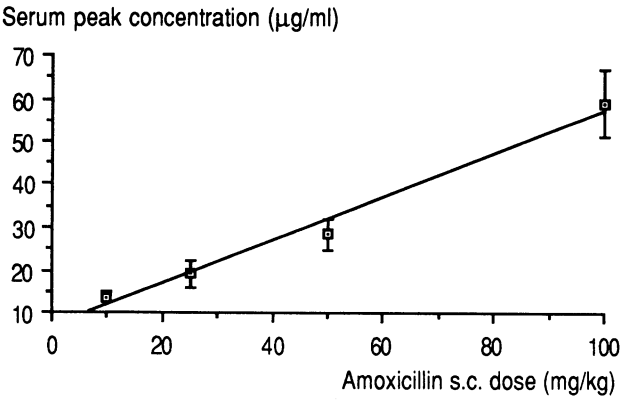

FIG. 2. Relationship between amoxicillin dosage and serum concentration at 0.5 h after a single s.c. dose ($r = 0.88$, $P < 0.01$). Values are representative means ± standard errors for five animals.

(7). In particular, gerbils seem to develop significant defenses against serotype 23 strains. Fulghum et al. (5) recovered no bacteria from ME effusions of gerbils 3 days after inoculation of 3×10^3 CFU of a serotype 23 per ear, whereas a 30-CFU inoculum of serotype 3 induced acute ME otitis. In our experiments, a 10^7-CFU inoculum of serotypes 23 and 19 induced purulent ME otitis in gerbils, and the disease course was similar with the three strains studied. Nevertheless, the acute phase of the disease was shorter than with a serotype 3 strain, and CSF involvement was not always lethal. In a previous study, we demonstrated that with a serotype 3 *S. pneumoniae* strain, meningeal complications are otogenic in this model and that meningeal infection is followed by CSF and blood dissemination (15). A similar study of the two serotype 23 strains used in the present work showed a similar extension of the infection (data not shown). CSF sampling was therefore performed to monitor, together with survival, the outcome of meningeal complications in control and treated animals. Survival of animals with positive CSF cultures is reported (rather than overall survival) to avoid including occasional deaths due to experimental accidents.

The large amount of bacteria injected in the ME bulla, the short course of the infection, and the risk of lethal meningeal complications argue for early treatment (2 to 4 h p.i.). Because of the rapid recovery of the animals, only a short treatment was possible to distinguish treatment efficacy from spontaneous bacterial clearance.

As expected in light of published data (10), ME infection induced by the penicillin-susceptible strain was eradicated after two administrations of 2.5 mg/kg 12 h apart. Indeed, this regimen must be sufficient to raise the concentration of amoxicillin in the ME effusion above the MIC for the infective strain for 24 h. Five mg/kg doses failed to clear the penicillin-resistant strains, but ME cultures were negative on day 2 after a 10-mg/kg dose (penicillin-resistant strain) and a 25-mg/kg dose (highly penicillin-resistant strain). Our pharmacokinetic study was limited by methodology and did not provide complete data on ME fluid penetration of amoxicillin at the doses used for treatment study. Nevertheless, available results taken together permit us to estimate by extrapolation the maximal concentrations of amoxicillin in ME effusion as 4 and 6 μg/ml (peak levels in serum, 13.5 and 19.2 μg/ml) after single 10- and 25-mg/kg s.c. doses, respectively. Under these conditions, amoxicillin levels in ME effusion must be maintained at more than 1 μg/ml for 5 h after a 10-mg/kg dose and more than 2 to 4 μg/ml for 2.5 to 4 h after a 25-mg/kg dose. For both penicillin-resistant and highly penicillin-resistant strains, the efficient dosages provided amoxicillin concentration above the MICs for only part of the time between two injections. High peak levels in the ME thus appear to be important to ensure bacterial eradication despite discontinuation of an amoxicillin level above the MIC.

With the SpRP-1 strain, regrowth was frequent at day 4 with all the regimens, including 2-day t.i.d. treatment. This phenomenon is not directly interpretable, since resistance of SpRP-1 was not found to be enhanced after discontinuation of amoxicillin treatment. However, differences in serotypes may be important. Better immunological competence vis-à-vis serotype 19 than serotype 23 could indeed favor therapeutic efficacy. Interestingly, differences in the immunogenicity of *S. pneumoniae* strains have been reported for young children (1).

Bacterial eradication at day 2 appears to be a good criterion for evaluating treatment efficacy in this model. Lethality associated with CSF involvement was limited

when the amoxicillin dosage resulted in bacterial clearance from the ME at day 2 p.i. This may have been due to a rapid decrease in bacterial counts in ME or to meningeal penetration by the drug.

At the dosages used in the treatment study, the pharmacokinetics of amoxicillin were situated in the range of clinical concentrations. Krause et al. (12) reported a maximum amoxicillin concentration of 5.6 μg/ml in ME effusion of children, 2 h after a single oral administration of 15 mg/kg, giving peak levels of 6 to 20 μg/ml in serum and an elimination half-life of approximately 50 min.

Under our experimental conditions, moderately increased dosages of amoxicillin do appear to cover a wide range of resistance in pneumococcal otitis. Nevertheless, these data must be interpreted cautiously in terms of clinical relevance and not be a priori extrapolated to all beta-lactams.

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