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# Amoxicillin Dose-Effect Relationship with *Streptococcus pneumoniae* in a Mouse Pneumonia Model and Roles of In Vitro Penicillin Susceptibilities, Autolysis, and Tolerance Properties of the Strains

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**We used a mouse model of pneumococcal pneumonia to assess the bactericidal effect of increasing doses of amoxicillin (AMX) against clinical strains with various susceptibilities to penicillin. Twelve strains that exhibited similar virulence in mice were selected. Three were penicillin susceptible (PS) (penicillin and AMX MICs = 0.01 to 0.03 μg/ml), three were intermediately resistant (PIR) (penicillin and AMX MICs = 0.5 to 1 μg/ml), and six were penicillin resistant (PR) (penicillin and AMX MICs = 1 to 8 μg/ml). Leukopenic Swiss mice were infected intratracheally with $10^7$ CFU of each strain. Treatment was initiated 3 h after infection and consisted of a single subcutaneous injection of AMX at doses ranging from 2.5 to 10 mg/kg (PS strains), 5 to 100 (PIR strains), and 25 to 3,000 (PR strains). Bacterial killing kinetics were recorded in the lungs over 9 h. The maximal log CFU reduction ($E_{max}$) was observed 3 h postinjection. The relation between $E_{max}$ and $\log_{10}$(dose/MIC) showed two populations. With seven strains (the three PS, the three PIR, and one of the six PR [MICs, penicillin/AMX = 4/1]) a good correlation was observed between $E_{max}$ and $\log_{10}$(dose/MIC) ($r = 0.772$; $P < 0.02$). A bactericidal effect equal to 3.5 $\log_{10}$ CFU was observed at a $\log_{10}$(dose/MIC) = 2. At this ratio, with the five other PR strains, $E_{max}$ varied from 0.4 to 1.6 $\log_{10}$ CFU. In brain heart infusion medium containing AMX at 50 times the relevant MIC, these five PR strains were tolerant in vitro. Treatment failure with AMX was found in vivo, with tolerant, highly resistant strains.**

*Streptococcus pneumoniae* is an important bacterial pathogen throughout the world, causing pneumonia, meningitis, and otitis media in adults and children. The emergence of penicillin-resistant and multiresistant strains has posed serious problems in the treatment of pneumococcal diseases, especially meningitis. Moreover, the tolerance of the strains, i.e., decreased susceptibility to the killing effect of beta-lactams, combined with increased levels of penicillin resistance among *S. pneumoniae* strains, probably explains some treatment failures. It has been reported (10, 11) that penicillin kills pneumococci through two distinct mechanisms: one triggers an autolytic enzyme, amidase, and the other, which is enzyme independent, involves the *cid* gene. Beta-lactam-tolerant strains have been reported (8), but their tolerance was mainly due to altered control of autolysin activity.

We assessed the effect of increasing doses of amoxicillin against *S. pneumoniae* strains in vivo, in a leukopenic mouse model of pneumonia, according to both in vitro susceptibility to penicillin and amoxicillin (AMX) and tolerance to beta-lactam antibiotics in vitro. Our aim was to determine if tolerance in pneumococcal strains influences the therapeutic efficacy of beta-lactam antibiotics in vivo.

(Part of this work was presented at the 34th Interscience Conference on Antimicrobial Agents and Chemotherapy [2].)

## MATERIALS AND METHODS

**Strains.** We used 12 clinical strains with similar experimental virulence (serotypes 6, 19, and 23) recovered from cerebrospinal fluid, sinus, ear, tracheal aspiration, bronchial aspiration, pleura, or blood. Three strains were penicillin susceptible (PS) (penicillin and AMX MICs = 0.01 to 0.03 μg/ml); three were intermediately resistant (PIR) (penicillin and AMX MICs = 0.5 to 1 μg/ml); and six were penicillin resistant (PR) (penicillin and AMX MICs = 2 to 8 and 1 to 8 μg/ml, respectively). The PIR and PR strains were of low experimental virulence, a characteristic related to their serotypes, as a strong link has been found between penicillin resistance, capsular type, and virulence (4, 5). The PS strains belonging to the same serotypes were also poorly virulent.

Two isogenic *S. pneumoniae* strains derived from strain RX were used: Cp 1015 (12) as the wild type, as its natural autolysis in response to calcium is well documented (15), and Cp 1095 (14, 15) bearing a mutation in the *lytA* gene which alters calcium-induced autolysis (autolysis-defective mutant).

**In vitro studies. (i) MICs and MBCs.** MICs and MBCs were determined in Mueller-Hinton infusion broth (Diagnostics Pasteur, Paris, France) supplemented with 5% sterile horse serum by using the tube dilution method (13). Each tube contained twofold dilutions of antibiotic and a final bacterial density of $10^6$ CFU/ml. Tubes were incubated for 18 h at 37°C in 10% $CO_2$–air. The MIC was defined as the lowest concentration of antibiotic at which no turbidity was visible to the naked eye. For the determination of MBC, 0.01-ml aliquots from tubes with no visible growth were plated onto Columbia agar with 5% sheep blood (Bio-Mérieux, Lyon, France) and incubated overnight at 37°C in 10% $CO_2$–air. The MBC was defined as the lowest concentration of antibiotic killing ≥99.9% of the original inoculum.

**(ii) Calcium-induced lysis.** Growth was monitored by optical density measurements at 400 nm on a Spectronic 401 spectrophotometer (Milton Roy). Autolysis was triggered by adding 1 mM calcium to the standard growth medium (CAT) at pH 7.8 and 37°C. Quantitative autolysis was given by the percent decrease in optical density at 400 nm, 6 h after the cultures reached the stationary phase (15).

**(iii) Beta-lactam-induced lysis.** To assess antibiotic-induced killing (tolerance studies), samples were diluted in brain heart infusion (BHI) broth containing 5% horse serum. When the culture (at 37°C) reached an absorbance of about 0.1 to 0.2 (corresponding to $10^8$ $\log_{10}$ CFU/ml), AMX or penicillin was added at concentrations of 5, 10, 25, and 50 times higher than the corresponding MIC. Viable bacteria were counted 6 h after adding the antibiotics, by plating appropriately diluted cultures on Columbia agar supplemented with 5% sterile sheep blood. Results are given for each strain as the log CFU reduction between the control and the 6-h culture in the presence of antibiotic. Moreillon et al. (10) have reported that wild-type cells ($Lyt^+$ $Cid^+$) (autolytic and nontolerant strains) lose 4 to 5 log units of viable counts after exposure to 20× MIC of penicillin for

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TABLE 1. Serotypes, microbiological data, virulence, and spontaneous autolysis of *S. pneumoniae* strains

| Strain | Serotype | Source of isolate[a] | MIC (μg/ml) | | $10^2$ lethal dose (CFU)[b] | Autolysis (pH 7.8) (%)[c] |
|---|---|---|---|---|---|---|
| | | | Penicillin | AMX | | |
| 47212 | 6 | TA | 0.01 | 0.01 | $0.9 \times 10^8$ | 49.0 ± 7.8 |
| 30923 | 6 | TA | 0.01 | 0.01 | $0.9 \times 10^8$ | 49.0 ± 7.8 |
| 52181 | 19 | BC | 0.01 | 0.03 | $1.2 \times 10^8$ | 44.0 ± 3.0 |
| 40225 | 23 | TA, BC | 0.5 | 0.5 | $8 \times 10^7$ | 43.0 ± 3.0 |
| 30189 | 6 | TA | 1 | 1 | $5.7 \times 10^8$ | 59.7 ± 4.0 |
| 54 B | 23 | Sinus | 1 | 1 | $5 \times 10^8$ | 61.0 |
| 54988 | 23 | Sinus | 4 | 1 | $5 \times 10^7$ | 26.0 |
| 40422 | 23 | CSF, BC | 2 | 2 | $9.3 \times 10^9$ | 59.6 |
| 12698 | 23 | TA | 4 | 2 | $4 \times 10^7$ | 53.0 ± 8.5 |
| 15986 | 19 | Ear | 8 | 4 | $1.1 \times 10^8$ | 71.2 ± 17 |
| 41375 | 23 | Pleura | 8 | 4 | $1.7 \times 10^8$ | 57.0 |
| 53681 | 23 | BA | 8 | 8 | $3.5 \times 10^8$ | 60.0 |
| Cp1015 | 6 | Mutant | 0.03 | 0.03 | $2.3 \times 10^8$ | 36.0 ± 8.7 |
| Cp1095 | 6 | Mutant | 0.03 | 0.03 | $2.2 \times 10^9$ | 94.0 ± 6.5 |

[a] TA, tracheal aspiration; BC, blood culture; CSF, cerebrospinal fluid; BA, bronchial aspiration.
[b] Experimental virulence in Swiss mice (mortality rates induced by intraperitoneal inoculation of $10^4$ CFU) was 0% with these strains.
[c] Spontaneous autolysis is expressed as percent residual optical density 12 h after initiation of the culture.

6 h. Triggering of amidase activity causes 1 log unit of viability loss, while the rest of the killing is linked to a *cid* marker.

**In vivo studies. (i) Experimental virulence.** Virulence assays were performed as described elsewhere (4, 5). Strains were grown at 37°C in BHI medium supplemented with 5% sterile horse serum. Mice were infected intraperitoneally with serial 10-fold dilutions of log-phase cultures. A strain is defined as virulent when an intraperitoneal inoculum of $10^4$ CFU, considered a minimal lethal dose, kills mice in one week or less. The scale was shifted to $10^7$ to $10^9$ CFU per mouse with the strains studied here, which were thus considered poorly virulent.

**(ii) Leukocyte depletion of mice.** To induce lethal pneumonia in mice with these poorly virulent *S. pneumoniae* strains, animals were depleted of leukocytes before infection. We induced sustained leukopenia in Swiss mice by means of three intraperitoneal injections of cyclophosphamide (Endoxan, Lucien Laboratories), 150 mg/kg daily, starting 5 days preinfection. This treatment reduced circulating leukocyte counts from 7,000 to about 1,000/$mm^3$ of blood.

**(iii) Mouse pneumonia model.** Leukopenic Swiss mice were infected intratracheally with $10^7$ CFU of *S. pneumoniae* (1). Treatment was started 3 h after infection and consisted of a single subcutaneous injection of AMX at doses ranging from 2.5 to 10 mg/kg (PS strains), 5 to 100 mg/kg (PIR strains), and 25 to 3,000 mg/kg (PR strains). Groups of five animals were killed by $CO_2$ asphyxiation before treatment and different times after treatment. Bacterial killing kinetics were recorded in the lungs over 9 h, relative to untreated control mice. Viable bacteria were counted in lung homogenates, before treatment and 1, 3, 6, and 9 h after starting treatment, by plating diluted homogenates on Columbia agar supplemented with 5% sterile sheep blood. Experiments were repeated at least twice with five mice in each control and treatment group.

**(iv) Pharmacokinetic studies with mice.** Pharmacokinetic parameters of amoxicillin were examined in infected Swiss mice. Lung and serum concentrations were determined after a single subcutaneous dose of 10, 25, 50, 100, 200, and 400 mg of AMX per kg. Serum and lung samples were collected from groups of three mice 0.5, 1, 2, 4, 6, and 8 h following drug administration. Animals were killed with $CO_2$ and exsanguinated by intracardiac puncture. Blood samples were centrifuged, and serum was collected. Lungs were harvested from exsanguinated mice, washed in sterile saline, weighed, and then homogenized in 1 ml of phosphate buffer (pH 7.8). Homogenates were centrifuged and supernatants were used for the assay. Antibiotic activities were determined by means of the agar well diffusion method, using *Escherichia coli* ATCC 3948 as the bioassay organism and Antibiotic Medium 1 (Difco Laboratories, Detroit, Mich.) as the growth medium. The detection limit of the assay is about 0.1 μg/ml or 0.1 μg/g, and the relative error is less than 10%.

**Statistical analysis.** The rates of penicillin and amoxicillin-induced killing in vitro were compared by multifactorial analysis of variance taking into account three groups, one including the PS and PIR strains, and two groups of resistant strains (tolerant and nontolerant strains). Multifactorial analysis of variance was also used to compare killing curves in vivo. The factors involved in this analysis were the strains, the ratio log (dose/MIC), and times after treatment. *P* values of 0.05 or less were considered significant.

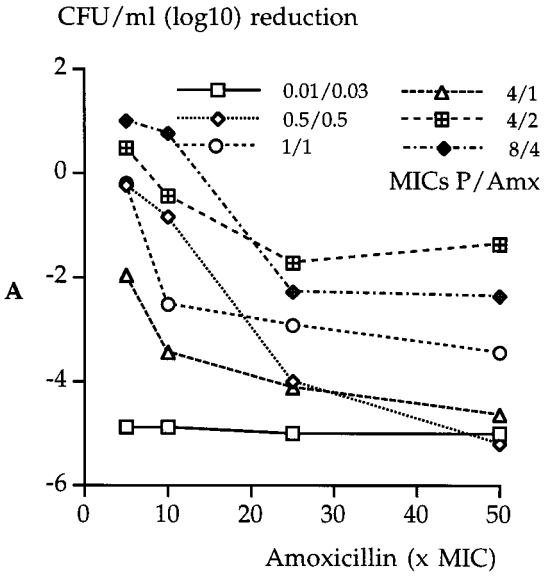
## RESULTS

**In vitro studies. (i) Autolysis.** The serotypes, penicillin and AMX susceptibilities, source (cerebrospinal fluid, sinus, ear, tracheal aspiration, bronchial aspiration, pleura, or blood), and autolysis of the strains are given in Table 1. In studies of autolysis in CAT medium (pH 7.8), the wild strain Cp 1015, considered naturally autolytic in the presence of calcium, showed a 64% decrease in turbidity (36% residual optical density), compared to 6% (94% residual optical density) with the autolytic defective mutant (Cp 1095, Lyt−). Among the clinical PR strains, 54988 autolyzed as well as the autolytic wild strain, while strain 15988 behaved as nonautolytic.

**(ii) Beta-lactam-induced killing.** Cultures of clinical strains in the exponential phase were treated with penicillin or AMX at 5-, 10-, 25-, and 50-fold the MIC, and the viable titer was monitored after 6 h of antibiotic contact. The rate of penicillin- or AMX-induced killing was 3 to 4 log units CFU for the PS and PIR strains (Fig. 1A and B). These strains are considered nontolerant. Among the PR strains, only one (54988) behaved differently from the others. No significant difference in beta-lactam killing was found between the PS-PIR strains and this PR strain, which was nontolerant. The five other PR strains were lysed by about one $\log_{10}$ CFU at 50-fold the relevant MIC (Fig. 1B) and were thus considered tolerant following the definition of Moreillon et al. (10). This feature is independent of their autolysis characteristics. The lack of killing with AMX at 5- and 10-fold the MIC excluded any paradoxical response of these strains to this antibiotic (Fig. 1A). There was a significant difference in beta-lactam killing in vitro between the group of PS-PIR strains and the group of tolerant PR strains ($P < 0.001$) and between the five tolerant PR strains and the nontolerant PR strain (54988) ($P < 0.001$).

**In vivo studies.** With the three PS strains studied (Fig. 2A), the maximal log CFU reduction ($E_{max}$) of about 3 log units was observed 3 h after the injection of AMX at 2.5 mg/kg, and was followed by a lengthy bacteriostatic effect (9 h). No bacterial regrowth was observed before 9 h, even though concentrations exceeded the MIC ($\Delta t$ MIC) for only 4 h. The peak concentration in serum ($C_{max}$) varied from 2 to about 10 μg/ml with 2.5 to 10 mg/kg, and the area under the curve (AUC) ranged from 1 to 11 μg/h/ml (Table 2).

With the three PI strains, the maximal log CFU reduction ranged from 2 to 3 log units and was observed after 3 h at a dose of 25 mg/kg, corresponding to a dose/MIC ratio of 25 or 50. This was also followed by a bacteriostatic effect (Fig. 2B).



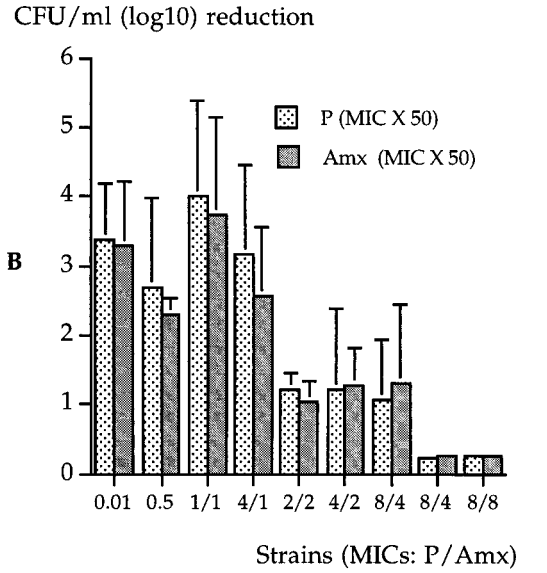
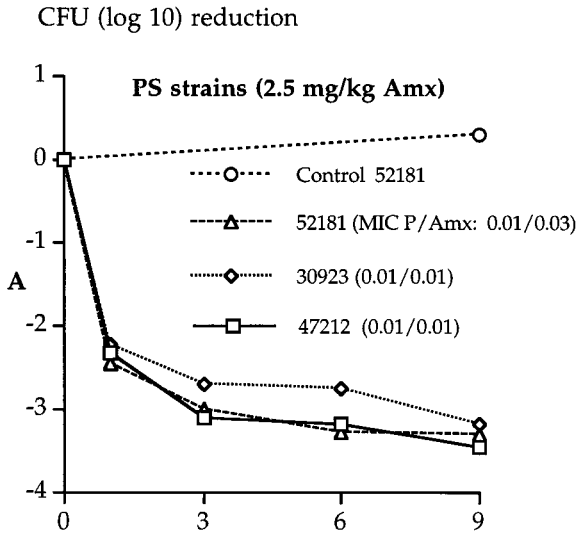




FIG. 1. Killing of PS, PIR, and PR strains in vitro: (A) by amoxicillin at 5, 10, 25, and 50-fold the relevant MICs (PS strain, 52181; PIR strains, 40225 and 54B; and PR strains, 54988, 12698, and 15986) and (B) by penicillin and AMX at 50-fold the relevant MICs (PS strain, 52181; PIR strains, 40225 and 54B; and PR strains, 54988, 40422, 12698, 15986, 41375, and 53681). Results are given for each strain as the $\log_{10}$ CFU reduction between control and the 6-h culture in presence of antibiotic.



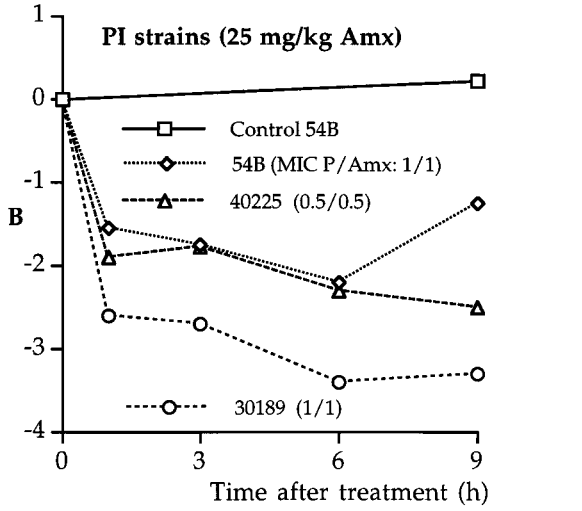




FIG. 2. Killing curves in vivo 1, 3, 6, and 9 h after single AMX injections at a dose of (A) 2.5 mg/kg against PS strains and (B) 25 mg/kg against PI strains.

At 25 and 50 mg/kg, $C_{max}$ values were 12 and 46 μg/ml, respectively; AUCs were 16 and 41 μg/h/ml, respectively; and $\Delta t$MIC was 2 h (Table 2).

The PR strains behaved differently according to their tolerance properties. With strain 54988 (Fig. 3A), which is not tolerant in vitro, $E_{max}$ ranged from 2.3 to 3.4 $\log_{10}$ CFU 3 h after one injection of AMX at doses ranging from 50 to 200 mg/kg (also corresponding to a dose/MIC ratio of 25 or 50, as with the PS and PIR strains). Among the five tolerant PR strains, only 12698 (Fig. 3B) showed an $E_{max}$ of 1.8 and 2.6 $\log_{10}$ CFU 6 h after one injection of AMX at doses of 200 and 400 mg/kg, respectively, followed by bacterial regrowth. However, we found a significant difference ($P < 0.02$) between this strain and the nontolerant strain (54988) in multifactorial analysis of variance including the dose/MIC ratios at all the time points. This difference remained significant ($P < 0.037$) with a dose/MIC ratio of 100, corresponding to 100 mg/kg with strain 54988 and 200 mg/kg with strain 12698. With the other PR-tolerant strains, an $E_{max}$ of ≤1 log CFU was obtained at AMX doses ranging from 100 to 3,000 mg/kg, corresponding to dose/MIC ratios of 300 and 400 (Fig. 4 and 5).

The relation between $E_{max}$ and $\log_{10}$(dose/MIC) (Fig. 6) revealed two populations. With seven nontolerant strains (the

TABLE 2. Pharmacokinetic parameters of AMX in the serum of infected Swiss mice following single subcutaneous doses[a]

| Dose (mg/kg) | Concn (μg/ml) after injection (h) | | | | | | $AUC_{0-8}$ (μg/h/ml) | $t_{1/2}$ (h) |
|---|---|---|---|---|---|---|---|---|
| | 0.5 | 1 | 2 | 4 | 6 | 8 | | |
| 2.5 | 2.2 ± 0.3 | 1.3 ± 0.3 | 0.48 ± 0.03 | 0.05 ± 0.01 | | | 1.45 | 0.32 |
| 5 | 3.3 ± 0.2 | 2.6 ± 0.43 | 0.65 ± 0.10 | 0.07 ± 0.02 | | | 2.36 | 0.31 |
| 10 | 6.4 ± 1.7 | 9.4 ± 4.3 | 0.44 ± 0.2 | 0.04 ± 0.02 | | | 11.0 | 0.40 |
| 25 | 12.1 ± 1.4 | 10.0 ± 5.3 | 1.5 ± 0.7 | 0.03 ± 0.01 | | | 16.0 | 0.36 |
| 50 | 46.6 ± 17.9 | 20.5 ± 9.3 | 1.5 ± 0.6 | 0.13 ± 0.08 | | | 41.2 | 0.41 |
| 100 | 66.7 ± 12.1 | 21.8 ± 3.1 | 3.6 ± 2.9 | 0.12 ± 0.03 | 0.07 ± 0.03 | 0.03 ± 0.01 | 55.5 | 0.39 |
| 200 | 118.1 ± 10.7 | 32.4 ± 6.5 | 4.9 ± 1.3 | 0.42 ± 0.04 | 0.12 ± 0.05 | 0.10 ± 0.04 | 91.8 | 0.41 |
| 400 | 211.8 ± 38.2 | 81.7 ± 18.6 | 12.9 ± 6.4 | 0.78 ± 0.06 | 0.42 ± 0.04 | 0.14 ± 0.03 | 189.0 | 0.43 |

[a] Values result from a pool of three samples of serum taken 0.5, 1, 2, 4, 6, and 8 h postdosing; $t_{1/2}$, elimination half-life; AUC, area under the curve.

three PS, the three PIR, and one PR strain: 54988) a good correlation was observed between $E_{max}$ and $\log_{10}$(dose/MIC) ($r = 0.772$; $P < 10^{-2}$). When all the strains were included in the analysis, no significant link was found between $E_{max}$ and $\log_{10}$(dose/MIC) ($r = 0.177$). The optimal bactericidal effect was observed with a dose/MIC ratio of about 100, i.e., $\log_{10}$(dose/MIC) = 2; $E_{max} = 3.5$ $\log_{10}$ CFU. Even with dose/MIC ratios of 300 and 400, an $E_{max} < 1$ $\log_{10}$ CFU was found with the highest PR strains. These strains were tolerant in vitro in BHI medium containing AMX at 50 times the relevant MICs.

## DISCUSSION

Moreillon et al. (10), using several mutants, defined two bactericidal targets for beta-lactams on pneumococci: one involves the triggering of a natural autolysin, *N*-acetylmuramoyl-L-alanine amidase, which causes 1 log unit of killing after exposure to 20-fold the MIC for 6 h, while the main mechanism, an enzyme-independent process that involves the Cid system, causes 3 to 4 log units of killing. This second bactericidal target has been identified in genetic terms. The tolerance trait appeared to be independent of resistance mechanisms, as there was no evidence of any alteration in penicillin-binding proteins. Moreover, lysis-defective mutants are found among susceptible clinical isolates, but a larger proportion has been found among penicillin-resistant mutants (11). In our study, most of the tolerant strains were among the resistant isolates, raising the question: is tolerance, either as an intrinsic characteristic of the strain or combined with penicillin resistance, an additive factor in therapeutic failure? The impact of tolerance has mainly been reported for experimental streptococcal endocarditis (6, 16), while no clinical studies of tolerant pneumococcal strains have been reported. This feature remains difficult to demonstrate in experimental pneumonia, as at the usual therapeutic doses investigated, the very high concentration/MBC ratio obtained may hinder the tolerance of penicillin-susceptible strains. Beta-lactam tolerance has been documented in vitro in several South African clinical isolates of penicillin-resistant pneumococci (8). However, this was shown by very limited lysis and cell wall breakdown after beta-lactam treatment at 20× MIC. Drug-specific tolerance was attributed to changes in the control of autolysin activity rather than survival after bactericidal doses of benzylpenicillin.

Analysis of beta-lactam killing in vivo by determining intrapulmonary bactericidal kinetics showed that increasing AMX doses had a maximal bactericidal effect against the PIR strains and one PR strain (54988). A linear relationship was found between $E_{max}$ and log dose/MIC when these strains and the susceptible strains were included in the analysis. In vitro,

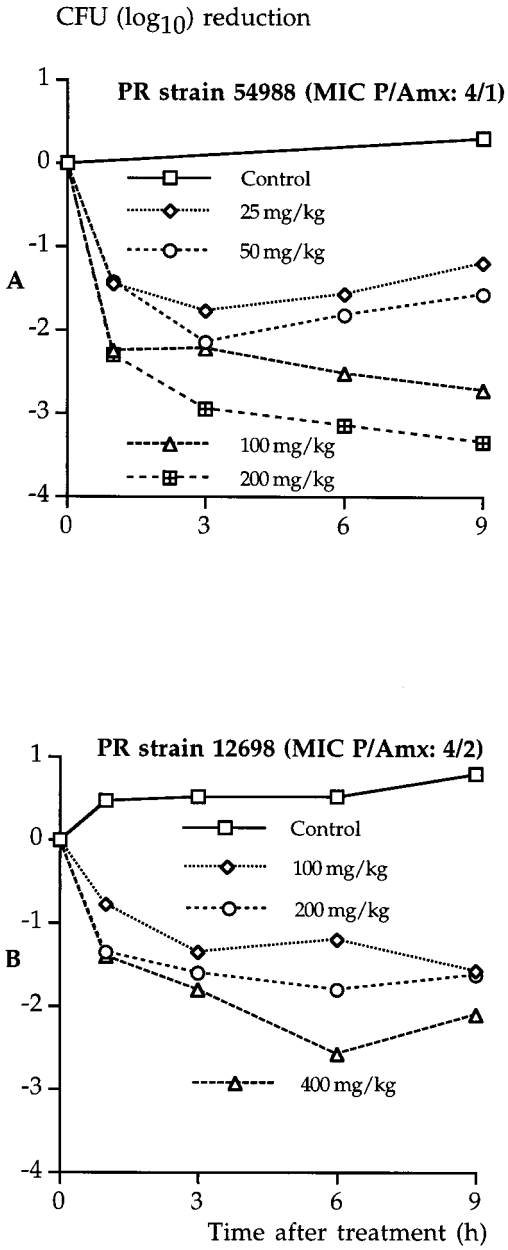



FIG. 3. Killing curves in vivo 1, 3, 6, and 9 h after single AMX injections at various doses against (A) a PR nontolerant strain (54988) and (B) a PR-tolerant strain (12698).

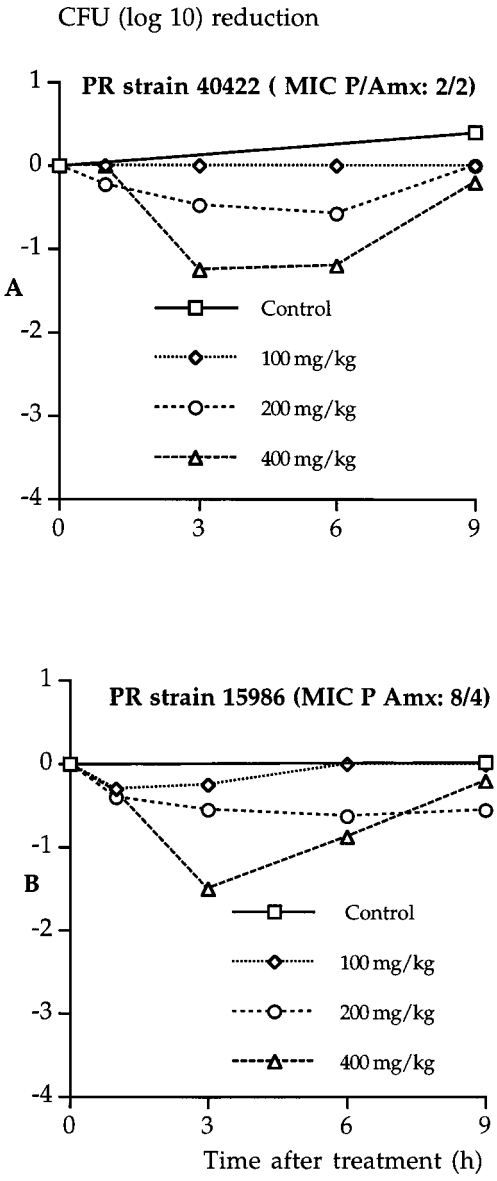



FIG. 4. Killing curves in vivo 1, 3, 6, and 9 h after AMX injections at various doses against PR-tolerant strains (A) 40422 and (B) 15986.

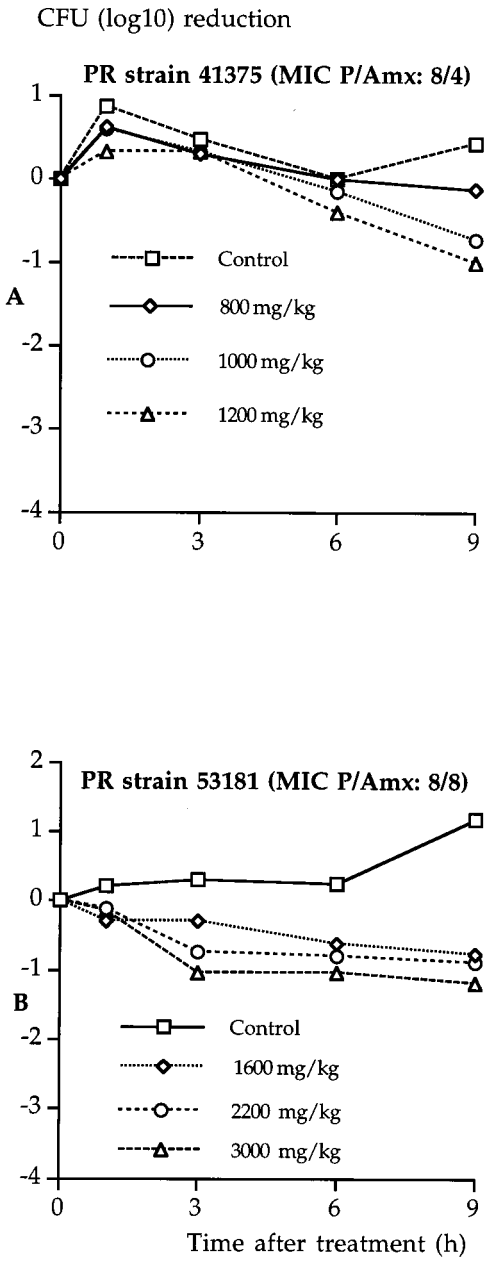



FIG. 5. Killing curves in vivo 1, 3, 6, and 9 h after AMX injections at various doses against PR-tolerant strains (A) 41375 and (B) 53181.

these strains had both a natural autolytic behavior intermediate between $Lyt^+$ and $Lyt^-$ mutants and a nontolerant phenotype. With the other resistant strains, high amoxicillin doses did not have a significant bactericidal effect in vivo even after 9 h of antibiotic contact and at dose/MIC ratios higher than 200. Only a bacteriostatic effect was observed. When these strains were included in the analysis, no significant link was found between $E_{max}$ and log dose/MIC. The highly penicillin-resistant strains had tolerant behavior in vivo, despite a natural autolytic feature. In vitro, the bactericidal effect was very poor even after 13 h of antibiotic contact at 50-fold the MICs. A relationship was found between increased MICs and both in vitro and in vivo tolerance. Treatment failure with AMX was thus encountered with tolerant, highly resistant strains. The tolerance profile may be important in clinical treatment failure. Alternative drugs which lack this characteristic could be used to avoid treatment failure. We have demonstrated (3) that imipenem overcomes tolerance and is active in vitro, and that vancomycin is bactericidal against a penicillin-resistant, tolerant, nonautolytic strain. Jabes and Tomasz (7) reported the lytic and bactericidal activity of a penem antibiotic against pneumococcal mutants exhibiting genetic tolerance. Moreover, we have shown that an antibiotic such as ceftriaxone with favorable pharmacokinetic parameters is effective against a tolerant PR clinical strain in vivo (9).

The implication of tolerance depends mainly on the site of infection. In pneumococcal pneumonia, alternative treatments to AMX might be found against tolerant strains. The adverse effects of bacterial tolerance would be most evident in immunocompromised patients or in severe invasive diseases such as meningitis and endocarditis in the normal host. For these in-

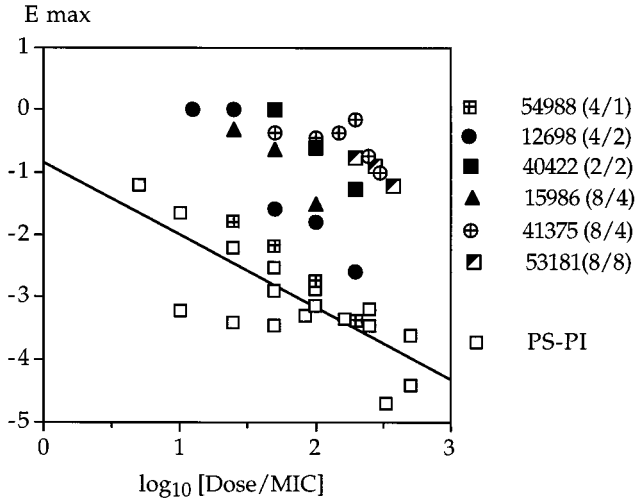



FIG. 6. Relation between maximal $\log_{10}$ CFU reduction ($E_{max}$) and $\log_{10}$(dose/MIC). For PS, PIR, and the nontolerant PR strain 54988, the equation of the relation is: $y = -1.163x - 0.826$; $r = 0.772$ ($P < 10^{-2}$).

fections confined to sites where an effective bactericidal action is needed for cure, the prevalence and clinical impact of tolerant pneumococci should be studied.

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