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Selection of Resistant *Streptococcus pneumoniae* during Penicillin Treatment In Vitro and in Three Animal Models

Jenny Dahl Knudsen,[1,2]* Inga Odenholt,[3] Helga Erlendsdottir,[4] Magnus Gottfredsson,[4,5]
Otto Cars,[6] Niels Frimodt-Møller,[2] Frank Espersen,[2] Karl G. Kristinsson,[4]
and Sigurdur Gudmundsson[5]

Department of Clinical Microbiology, Rigshospitalet,[1] and Microbiological Research and Development, Statens Serum Institut,[2] Copenhagen, Denmark; Department of Infectious Diseases, University Hospital, Malmö, Sweden[3]; Departments of Microbiology[4] and Internal Medicine,[5] Landspitalinn (University Hospital), Reykjavík, Iceland; and Department of Infectious Diseases, University Hospital, Uppsala, Sweden[6]

Pharmacokinetic (PK) and pharmacodynamic (PD) properties for the selection of resistant pneumococci were studied by using three strains of the same serotype (6B) for mixed-culture infection in time-kill experiments in vitro and in three different animal models, the mouse peritonitis, the mouse thigh, and the rabbit tissue cage models. Treatment regimens with penicillin were designed to give a wide range of $T_{>MIC}$s, the amounts of time for which the drug concentrations in serum were above the MIC. The mixed culture of the three pneumococcal strains, 10^7 CFU of strain A (MIC of penicillin, 0.016 μg/ml; erythromycin resistant)/ml, 10^6 CFU of strain B (MIC of penicillin, 0.25 μg/ml)/ml, and 10^5 CFU of strain C (MIC of penicillin, 4 μg/ml)/ml, was used in the two mouse models, and a mixture of 10^5 CFU of strain A/ml, 10^4 CFU of strain B/ml, and 10^3 CFU of strain C/ml was used in the rabbit tissue cage model. During the different treatment regimens, the differences in numbers of CFU between treated and control animals were calculated to measure the efficacies of the regimens. Selective media with erythromycin or different penicillin concentrations were used to quantify the strains separately. The efficacies of penicillin in vitro were similar when individual strains or mixed cultures were studied. The eradication of the bacteria, independent of the susceptibility of the strain or strains or the presence of the strains in a mixture or on their own, followed the well-known PK and PD rules for treatment with β-lactams: a maximum efficacy was seen when the $T_{>MIC}$ was >40 to 50% of the observation time and the ratio of the maximum concentration of the drug in serum to the MIC was >10. It was possible in all three models to select for the less-susceptible strains by using insufficient treatments. In the rabbit tissue cage model, a regrowth of pneumococci was observed; in the mouse thigh model, the ratio between the different strains changed in favor of the less-susceptible strains; and in the mouse peritonitis model, the susceptible strain disappeared and was overgrown by the less-susceptible strains. These findings with the experimental infection models confirm the importance of eradicating all the bacteria taking part in the infectious process in order to avoid selection of resistant clones.

Antimicrobial resistance in clinical isolates of *Streptococcus pneumoniae*, pneumococci, has spread throughout the world and gained high prevalence in many countries (1, 19, 20). The pneumococcus remains one of the leading causes of community-acquired bacterial infections, and severe pneumococcal infections such as pneumonia and meningitis have high morbidity and mortality rates (17). The high prevalence of multidrug-resistant pneumococci has created problems in treating these infections, and there is a renewed interest in treatment strategies and in the ecology of these strains (2, 6, 14). Individuals may be colonized with several strains of the same pathogen, such as various strains of pneumococci, with different antibiotic susceptibilities, although one serotype seems to dominate in the majority of infections (6, 15, 16). Antimicrobial therapy providing a certain pharmacokinetic (PK) profile may be sufficient for eradication of some of the more-susceptible organisms but may not affect others and may therefore be selective of resistant clones or subpopulations, creating a selective window (3, 4). Relatively few organisms from a resistant

clone or subpopulation could therefore be selected during treatments that affect only the predominant populations (3, 4).

Research with animal models and single strains with various penicillin susceptibilities indicates that by optimizing the dosing of penicillin, the drug may still be used effectively even against non-penicillin-susceptible pneumococci (7, 12, 13; V. Magnusson, H. Erlendsdottir, K. G. Kristinsson, and S. Gudmundsson, Abstr. 35th Intersci. Conf. Antimicrob. Agents Chemother., abstr. A89, p. 17, 1995; A. Thoroddsen, T. Asgeirsson, H. Erlendsdóttir, and S. Gudmundsson, Abstr. 37th Intersci. Conf. Antimicrob. Agents Chemother., abstr. B-5b, p. 27, 1997). However, studies on the effects of antimicrobials on mixed bacterial infections in experimental animal models are lacking.

A recent study examined the pharmacodynamic (PD) responses to penicillin treatment of infections with penicillin-susceptible and -resistant strains of the same serotype with the use of comparable penicillin dosing regimens in four different animal models: peritonitis, pneumonia, and thigh infection in mice and tissue cage infection in rabbits (7). Similar efficacies were observed in all models with comparable treatment regimens (7).

Using the same bacterial strains in several different animal models eliminates the effects of different strain characteristics

* Corresponding author. Mailing address: Damstein 7B, DK-2720 Vanloese, Denmark. Phone: 45 3874 9930. Fax: 45 3874 9932. E-mail: jdk@ssi.dk.

TABLE 1. PK and PD parameters of the various treatment regimens

Group	Dose (mg/kg of body weight)	No. of times administered (h after start of regimen)	C_{max} (µg/ml)	$T_{>MIC}$ (h) for strain[a]:			C_{max}/MIC[b] for strain:		
				A	B	C	A	B	C
Mouse models[c]									
1	0.12	1 (0)	0.12	0.75	0	0	7.50	0.48	0.03
2	0.12	2 (0, 3)	0.12	1.50	0	0	7.50	0.48	0.03
3	0.12	3 (0, 2, 4)	0.12	2.25	0	0	7.50	0.48	0.03
4	0.12	6 (0, 1, 2, 3, 4, 5)	0.12	4.50	0	0	7.50	0.48	0.03
5	2	1 (0)	2	1.20	0.75	0	125	8	0.5
6	2	2 (0, 3)	2	2.40	1.50	0	125	8	0.5
7	2	3 (0, 2, 4)	2	3.60	2.25	0	125	8	0.5
8	2	6 (0, 1, 2, 3, 4, 5)	2	>6.00	4.50	0	125	8	0.5
9	32	1 (0)	32	2.57	1.38	0.75	2000	128	8
10	32	2 (0, 3)	32	5.22	2.82	1.50	2000	128	8
11	32	3 (0, 2, 4)	32	>6.00	4.20	2.25	2000	128	8
12	32	6 (0, 1, 2, 3, 4, 5)	32	>6.00	>6.00	4.50	2000	128	8
Control			0	0	0	0	0	0	0
Rabbit tissue cage model[d]									
1	3	1 (−1)	0.33	16	1	0	20.63	1.32	0.08
2	15	1 (−1)	1.39	>24	11	0	86.88	5.56	0.35
3	30	1 (−1)	2.85	>24	15	0	178.13	11.40	0.71
4	75	1 (−1)	11.11	>24	20	6	694.38	44.44	2.78
Control			0	0	0	0	0	0	

[a] Values on which the regimens were based are boxed.
[b] MICs were 0.016, 0.25, and 4 µg/ml for strains A, B, and C, respectively.
[c] PK and PD parameters were calculated.
[d] PK and PD parameters were measured during the experiments.

and thus provides a better understanding of the importance of the PK and PD parameters at the different sites of infection (7; Thoroddsen et al., 37th ICAAC).

Using the rabbit tissue cage model, the mouse peritonitis model, and the mouse thigh model, now with a mixed-culture infection with three strains of pneumococci, we studied the PD responses to penicillin treatment. The focus of this work was especially on the properties selective of resistant strains. The treatment regimens were designed to give a wide range of $T_{>MIC}$s, the amounts of time for which the drug concentrations in serum were above the MIC.

(This work was presented in part at the 40th Interscience Conference on Antimicrobial Agents and Chemotherapy, Toronto, Canada, 17 to 20 September 2000 [abstract 2246].)

MATERIALS AND METHODS

Bacterial strains. Three clinical isolates of pneumococci from Iceland were used, all belonging to serotype 6B. Strain A (A-2000) was susceptible to penicillin (MIC = 0.016 µg/ml) and resistant to erythromycin (MIC > 128 µg/ml); the MICs of penicillin for strain B (9506-07126) and strain C (BCC-67) were 0.25 and 4 µg/ml, respectively, and both strains were susceptible to erythromycin (MIC ≤ 0.03 µg/ml). The minimal bactericidal concentration of penicillin was greater than or equal to two times the MIC for all strains.

Bacterial suspensions were always made fresh from an overnight culture, and the inocula were always controlled by quantitative cultivation.

A mixed culture was used in all models and in vitro; the inocula were intended to consist of approximately 10^7 CFU of strain A/ml, 10^6 CFU of strain B/ml, and 10^5 CFU of strain C/ml in the mouse models and in vitro and approximately 10^5 CFU of strain A/ml, 10^4 CFU of strain B/ml, and 10^3 CFU of strain C/ml in the rabbit model.

MICs. The MICs were determined by the E test (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions.

Antibiotic agent. Benzylpenicillin (penicillin G) for treatment of animals was obtained from Astra AB, Södertälje, Sweden. Dilutions were made fresh for each experiment by using distilled water or phosphate-buffered saline.

Time-kill studies. For each strain, time-kill curves were determined three times with inocula of 10^6 and 10^7 CFU/ml and with concentrations of 4 and 16 times the MIC and 16 µg/ml, respectively. Time-kill studies were performed with the mixed culture by using the penicillin G concentrations of 0.064, 0.50, and 16 µg/ml, respectively.

Animals. Female NMRI mice approximately 8 weeks old, weighing 30 ± 2 g, were used for the mouse thigh infection model. For the mouse peritonitis model, CF1 female mice approximately 8 weeks old, weighing 30 ± 2 g, were used. Three- to four-month-old female New Zealand White rabbits, weighing 2.5 to 3.2 kg, were used for the tissue cage model.

Animal models. All the animal models used were approved by the local animal ethics committees in our respective countries.

Before the three strains were used in the different animal models, as described below, the growth of each strain with different inocula was tested in the CF1 mice. Four mice were inoculated intraperitoneally with 10^3, 10^5, or 10^7 CFU of one of the three strains, respectively. Three and six hours after inoculation, two mice from each group were bled and bacterial counts in blood were determined as described below for the mouse peritonitis model.

The lethality of the mixed-culture infection was tested on a group of five mice, and all died within 24 h.

Mouse thigh infection. Bacterial suspensions were prepared from fresh overnight cultures (made from frozen stock cultures) on 5% blood agar plates. The bacteria were grown in brain heart infusion broth with 10% horse serum for 6 h at 35°C. The mice were infected by injecting 0.1 ml of the mixed culture in brain heart infusion broth with 10% horse serum into the thighs (9). Antibiotic therapy was initiated 2 h after thigh inoculation. Penicillin was administered subcutaneously in the neck regions in a volume of 0.2 ml per dose. Each treatment group comprised three mice, and five inoculated, untreated control mice were included in all trials.

At the end of the experiment, after 6 h of treatment, the animals were sacrificed by cervical dislocation and the thighs were removed and homogenized (tissue homogenizer; Omni, Gainesville, Va.) in saline with β-lactamase (penase; 100,000 U/ml) to a total amount of 10 ml to neutralize residual antibiotics. Bacterial densities in the thighs were determined by plating serial 10-fold dilutions on different plates (see "End points" below). The lowest detection level for the viable counts in the thighs was 100 CFU per thigh.

Mouse peritonitis. Bacterial suspensions were prepared from fresh overnight cultures (made from frozen stock cultures) on 5% blood agar plates. The inoculum for the mouse peritonitis model was prepared immediately before use by
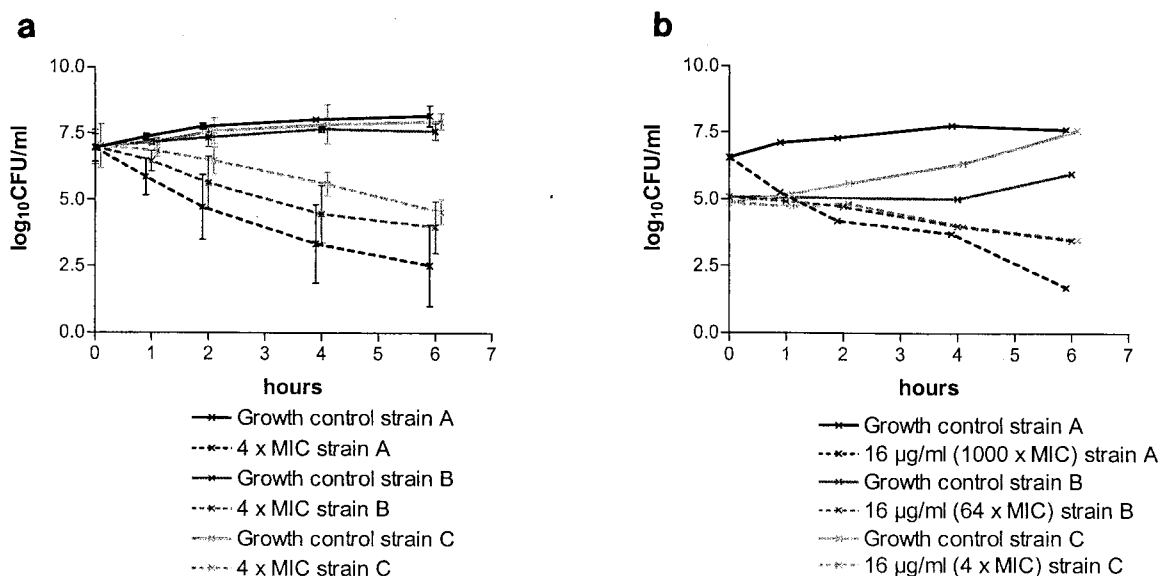
FIG. 1. Time-kill curves for strains A, B, and C tested separately (a) and as a mixed culture (b). Curves are drawn based on the means of results from three experiments, with error bars showing standard deviations.

suspending the bacteria in sterile beef broth media; adjusting the suspension to an optical density at 540 nm of 0.5, which gave a density of approximately 10^8 CFU/ml; and subsequently diluting the suspension with saline. The size of the inoculum was determined by viable counts on 5% blood agar. The mice were injected intraperitoneally with 0.5 ml of the pneumococcal suspension, resulting in bacteremia within 1 h of inoculation (12). Antibiotic therapy was initiated 1 h after inoculation. Penicillin was administered subcutaneously in the neck regions in a volume of 0.1 ml per dose. Each treatment group comprised three mice. Five inoculated, untreated control mice were included in all trials. The effects of the various treatment regimens were determined after 6 h of treatment. Blood samples were obtained by periorbital cutting after the mice were anesthetized with CO_2. Blood samples were immediately diluted 10-fold in saline, and 20-μl aliquots were plated in spots onto different agar plates (see "End points" below), with subsequent counting of colonies after incubation overnight at 35°C in ambient air. The lowest detection level for bacterial counts in blood samples was 50 CFU/ml and that for counts in the peritoneal fluid was 250 CFU/ml.

Rabbit tissue cage. Rabbits were anesthetized with 0.5-ml intramuscular injections of fentanyl-fluanisone (Hyponorm) followed by disinfection of the backs of the rabbits with 70% alcohol and administration of local anesthetic (lidocaine, 40 mg/ml) (18). A 5-cm incision was then made in the midline of each animal, and four well-separated pouches were bluntly dissected in the subcutaneous layer. In each pouch, an autoclaved cylindrical steel net cage with a volume of 4 ml was implanted. The incision was closed with sutures. To reverse the anesthesia, the rabbits were given 0.3 to 0.4 ml of naloxone hydrochloride (Narcante) at a concentration of 0.4 mg/ml through intravenous needles in the ear veins. Three to four weeks after the implantation, the tissue cages had sealed with a thin layer of connective tissue and filled with clear, yellowish tissue cage fluid. The bacterial suspensions used in the rabbit tissue cage model were grown in Todd-Hewitt broth for 6 h at 37°C in 5% CO_2-supplemented air, resulting in an inoculum of approximately 5×10^8 CFU/ml. The bacteria, when in the logarithmic phase, were subsequently diluted in phosphate-buffered saline to the intended inoculum concentration and mixed (10^5 CFU of strain A/ml, 10^4 CFU of strain B/ml, and 10^3 CFU of strain C/ml), and thereafter 0.4 ml of the mixture was injected into each of the four cages. The rabbits were treated with intravenous injections in the ear veins. Samples of 0.2 ml from the cages were obtained by percutaneous aspiration every third hour up to 12 h and, in addition, after 24 h. Samples from the two left and right cages were pooled together before plating. To avoid antibiotic carryover, the samples were treated with β-lactamase (penase; 100,000 U/ml) and if necessary diluted in phosphate-buffered saline before being seeded onto the different agar plates (see "End points" below). The lowest detection level for bacterial counts in tissue cage samples was 50 CFU/ml.

PK of penicillin in mice and rabbits. The PK of penicillin G in mice was studied previously (7, 9, 12), and PK data were obtained for the rabbit model during the experiments in the present study. For the mouse models, a half-life ($t_{1/2}$) in serum of 12.5 min and a volume of distribution of 1 liter/kg were used (7, 9, 12).

The levels of protein binding in serum were formerly determined to be 8.5% (0 to 17%) in mouse serum and 24% (13 to 36%) in rabbit serum (7). These relatively low levels of protein binding were considered insignificant and therefore not included in the calculations.

Treatment regimens. The design of the regimens for the animal models was based upon results from previous PK studies (7, 9, 12), and doses and PK and PD parameters are given in Table 1.

Twelve different penicillin treatment regimens were carried out in the mouse models, with three mice per treatment group for each mouse model, and four different treatment regimens were used in the rabbit model, with two rabbits with four tissue cages each per treatment group (Table 1).

Groups of untreated control animals were always included.

The twelve different regimens in the mouse models were designed to have $T_{>MIC}$s of 12.5, 25, 37.5, and 75% of the observation time for each of the three strains (Table 1).

Since the maximum concentration of penicillin in serum (C_{max}) in the rabbit tissue cage model is reached approximately 1 h after injection, antibiotic therapy was initiated 1 h before inoculation (7, 18).

End points. The times used as end points were 6 h in the mouse models and 24 h in the rabbit model, as used previously (7).

The differences in numbers of CFU between treated and control animals were calculated to measure the efficacies of the treatment regimens. For all the models, the samples were drawn and immediately cultivated undiluted (100 μl on one plate) or diluted 10-fold in 20-μl spots plated onto various plates for measurement of the total numbers of CFU and the numbers of CFU of each strain. Plates with the following media were used: (i) 5% blood agar for counting the total numbers of CFU of strains A, B, and C; (ii) 5% blood agar with 1 μg of erythromycin/ml for counting CFU of strain A; (iii) 5% blood agar with 0.062 μg of penicillin/ml for counting CFU of strains B and C; (iv) 5% blood agar with 1 μg of penicillin/ml for counting CFU of strain C; (v) 5% blood agar with 8 μg of penicillin/ml, used only for the mouse peritonitis experiments to ensure that no new resistant clone developed.

Only counts between 10 and 1,000 were included in the calculations of the numbers of CFU per milliliter.

Statistical methods. The nonparametric Spearman test was used to study the correlations between efficacy and the PK and PD parameters. The Dunn multiple comparison test was used to compare efficacies of regimens grouped by PK and PD parameters. P values of less than 0.05 were considered significant.

RESULTS

Penicillin was bactericidal to similar extents in vitro when individual strains and mixed cultures were studied (Fig. 1). The extents of killing were similar at 4 and 16 times the MIC for

TABLE 2. Bacterial generation times in vitro and in vivo

Strain	Bacterial generation time(s) (min)		
	In vitro[a]	In rabbit tissue cages[b]	In mouse blood after peritoneal inoculation[c]
A	70, 92	35	23, 33, 38
B	133, 171	44	35, 26, 40
C	57, 113	41	102, 60, 194

[a] For the time period from 1 to 6 h, when start inocula were 10^6 (data not shown) and 10^7 (Fig. 1a) CFU/ml, respectively.

[b] For the time period from 1 to 9 h after inoculation.

[c] For the time period from 3 to 6 h after inoculation with inocula of 10^3, 10^5, and 10^7 CFU, respectively.

each strain alone, but among the kill rates for the different strains, the highest was that for strain A, followed by those for strains B and C, which may be explained by different growth rates (Fig. 1; Table 2). The in vitro generation times were slightly different, which was attributed to the sizes of the start inocula; i.e., when the start inoculum was approximately 10^7 CFU/ml (Fig. 1a), the generation times were 92, 171, and 113 min for strains A, B, and C, respectively, and when the start inoculum was approximately 10^6 CFU/ml (data not shown), the generation times were 70, 133, and 57 min, respectively (Table 2). The pneumococcal cultures in vitro seemed to reach a maximum density of approximately 10^8 CFU/ml after 6 h.

The growth rate of strain B was lower when tested in the mixed-culture time-kill experiments than it was when strain B was tested alone, but the killing rates were similar (Fig. 1b).

In the mouse peritonitis model, all three strains separately showed exponential growth independent of inoculum size, although the generation times for the strains were different—the growth of strain C was slower in mice (Table 2). When the mixed culture was inoculated into mice, strain B was the one with the slowest growth during the period from 0 to 6 h in the untreated mice during the experiments in both mouse models (Fig. 2), a result similar to that seen for strain B in the in vitro time-kill study (Fig. 1b).

The killing efficacies differed in the two mouse models (Fig. 2). The selection of the resistant strains during the different treatments was more obvious in the mouse peritonitis model than in the thigh model. In the peritonitis model, it was possible to select the resistant strains by dosing (Fig. 2). It was not possible to eradicate the susceptible strain A in the thigh model, although the relative amount of the susceptible strain decreased during the treatments (Fig. 2). As seen in Fig. 2, the killing of the bacteria was more dependent on the size of the dose of penicillin than on the dosing regimen, as the change in bacterial counts seemed to follow the shift in doses more than the shift in regimens. The parameters $T_{>MIC}$ and C_{max}/MIC
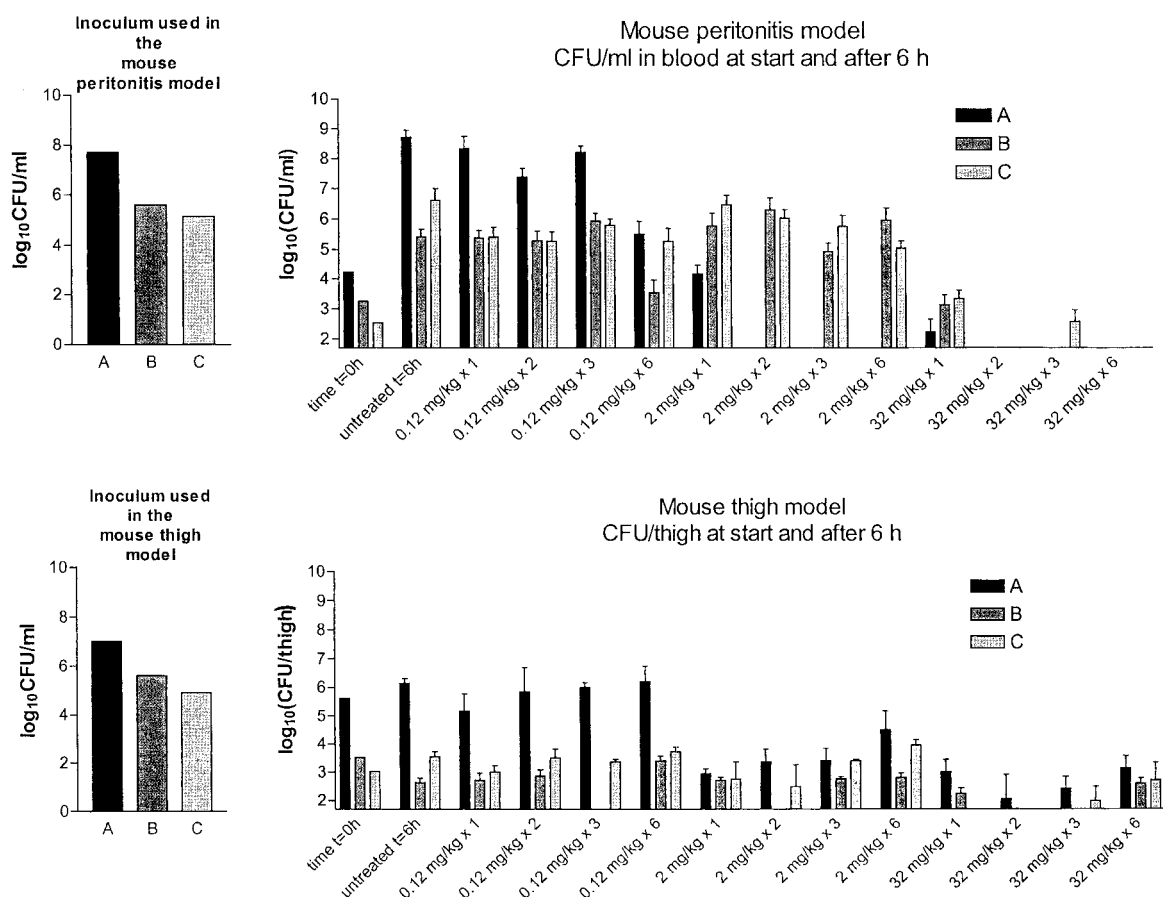

FIG. 2. Bacterial counts in inocula and in blood at the start and after 6 h of treatment in the mouse peritonitis and thigh models. Columns show means of results for three mice, and bars show standard deviations. Values following multiplication signs indicate the numbers of times that the doses were administered.
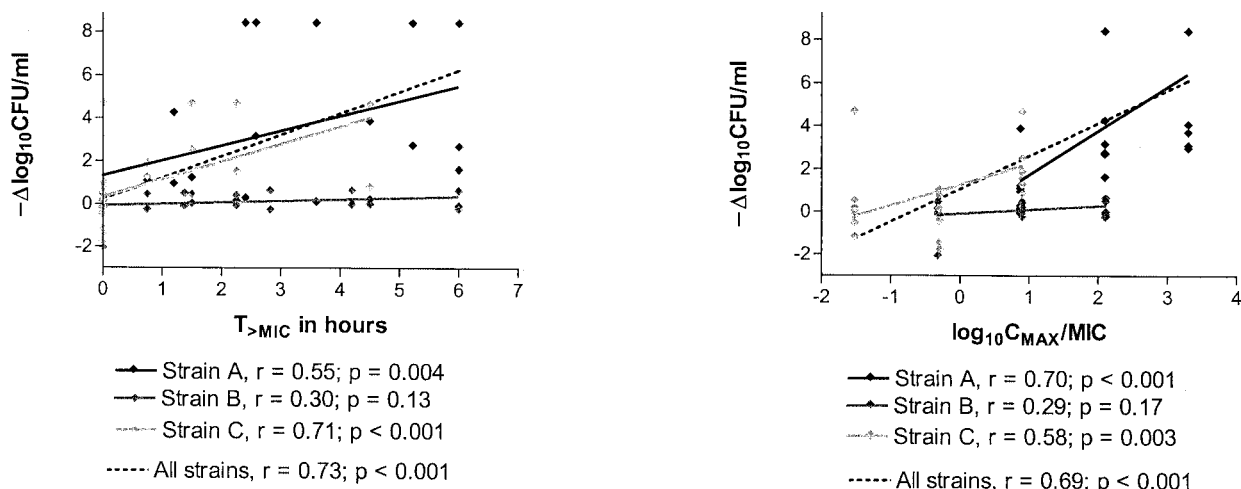
FIG. 3. Correlations between efficacy and $T_{>MIC}$ and between efficacy and the C_{max}/MIC ratio in the mouse models. y axes show the differences in numbers of CFU between treated animals and untreated controls.

both correlated significantly with killing efficacy when all data were collected, but for strain B tested separately, none of the parameters correlated significantly (Fig. 3). When the data from the thigh model and the peritonitis model were analyzed separately, no major differences were found. When the efficacy data were analyzed according to groups of regimens, given a $T_{>MIC}$ threshold of more or less than 40% (i.e., 2.4 h) or a C_{max}/MIC threshold of more or less than 10 (Fig. 4), the efficacy was found to be significantly higher when both these parameters were above these thresholds than when both parameters were below. Few of the regimens had only one of the parameters above the thresholds, and although the differences are not significant, these regimens were less efficacious (Fig. 4).

The correlation between $T_{>MIC}$ and $\log_{10}(C_{max}/MIC)$ for all regimens in the mouse models was highly significant (Spearman's rho = 0.84; $P < 0.001$).

In the rabbit tissue cage model, the strains behaved more similarly; all three strains showed fast exponential growth in the rabbit model in the first 9 h after challenge and had similar generation times (Table 2; Fig. 5). A leveling off to a slight decrease of counts was observed after 9 h, but the relative amount of each strain in the mixed cultures seemed to be maintained (Fig. 5).

In the rabbit model, selection of the resistant strains was seen and regrowth occurred during insufficient treatments (Fig. 5). The killing effect was found to be significantly correlated with both $T_{>MIC}$ and the C_{max}/MIC ratio when data for all strains were accumulated (Fig. 6). The correlation between $T_{>MIC}$ and $\log_{10}(C_{max}/MIC)$ for the regimens in the rabbit tissue cage model was highly significant (Spearman's rho = 0.96; $P < 0.001$). It was not possible to compare the efficacies according to the PK and PD parameters with the Dunn's multiple comparison test as it was in the mouse models, but only if $T_{>MIC}$ was more than 11 h (i.e., 46%) and the C_{max}/MIC ratio was more than 10 was a maximal killing effect seen (Fig. 6). Strain B showed regrowth during the regimen of 30 mg/kg of body weight, although the $T_{>MIC}$ was 15 h (i.e., 63%) and the C_{max}/MIC ratio was 11; on the other hand, strain C was erad-

icated by the 75-mg/kg regimen, although the $T_{>MIC}$ was only 6 h (i.e., 25%) and the C_{max}/MIC ratio was only 2.8 (Fig. 5; Table 1). When 12 h was used as the end point for the rabbit tissue cage model, the thresholds for efficacy were approximately 6 h (i.e., 50%) for $T_{>MIC}$ and approximately 10 for the C_{max}/MIC ratio (data not shown).

No development of further resistance to penicillin was found during the treatments, as no colonies were found on the blood agar plates with 8 μg of penicillin/ml.
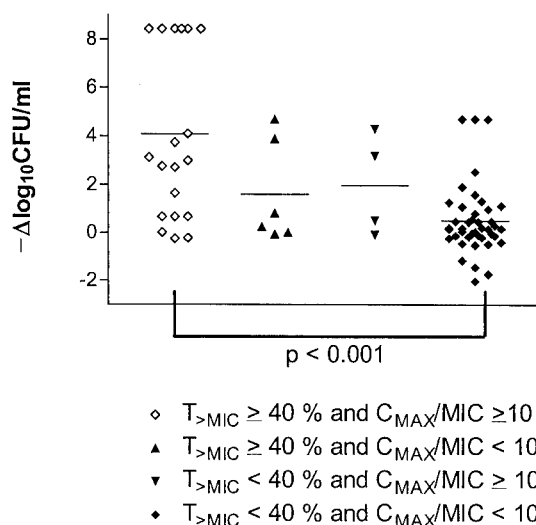

FIG. 4. Efficacies of the regimens in the mouse models grouped according to PK and PD parameters. Efficacies of regimens in which $T_{>MIC}$ was at or above 40% and the C_{max}/MIC ratio was at or above 10 were significantly higher than those of regimens in which only one of the parameters was above the threshold value. No other statistically significant differences were found between the groups. y axes show the differences in numbers of CFU between treated animals and untreated controls.
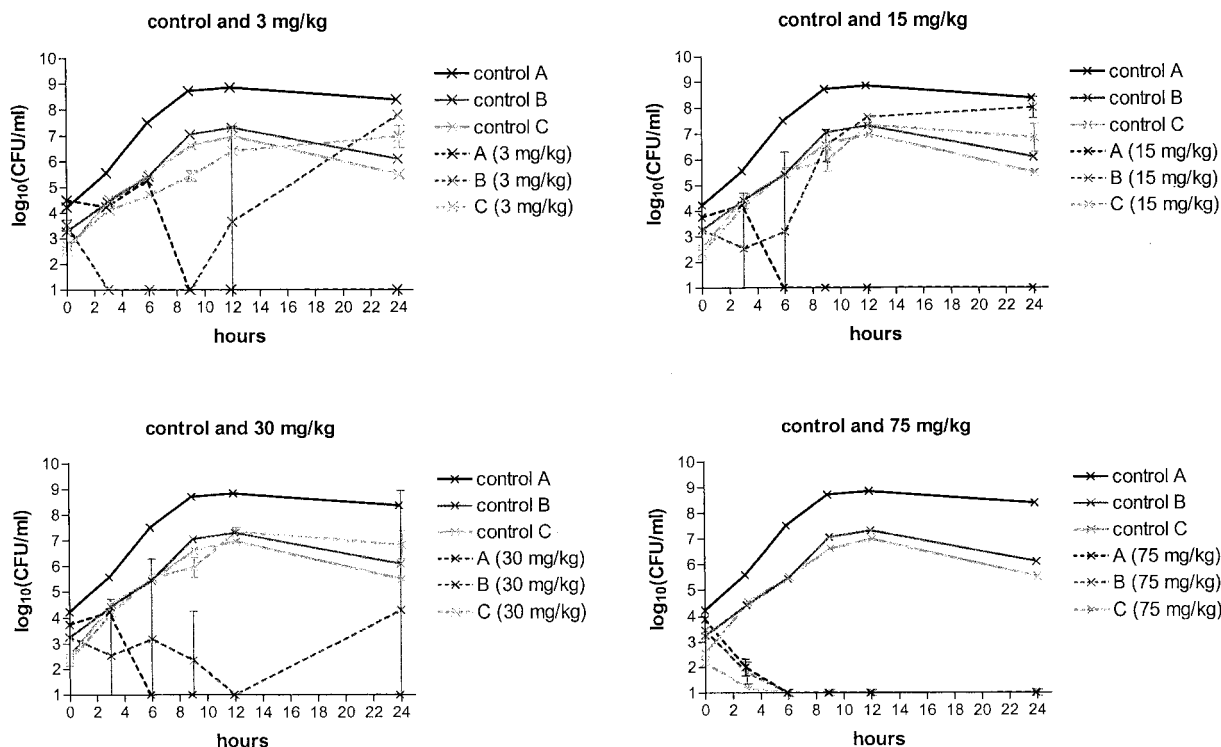
FIG. 5. Bacterial counts in the rabbit tissue cages at specific times after challenge. Curves are drawn based on means of results from two experiments, with bars showing standard deviations.

DISCUSSION

Antibiotic dosing regimens should be designed not only to optimize the elimination of the infecting pathogen but also to minimize the risk of emergence of resistant bacteria. Failure to eradicate resistant bacteria increases both the risk of clinical failures and the risk of spreading resistant clones throughout populations. Little is known about the selective properties of different antibiotic regimens in a mixed culture of bacteria with different antibiotic susceptibilities. The normal nasopharyngeal flora of patients simultaneously comprises different types of bacteria and may include several members of the same species, e.g., pneumococci, showing variable resistance patterns. A certain dosage regimen may select more-resistant strains even when they, at the time of treatment, constitute a minority of the original population.

It is hoped that using the same pneumococcal strains in a mixture in three different animal models can provide reliable information since parameters of importance must be universal.
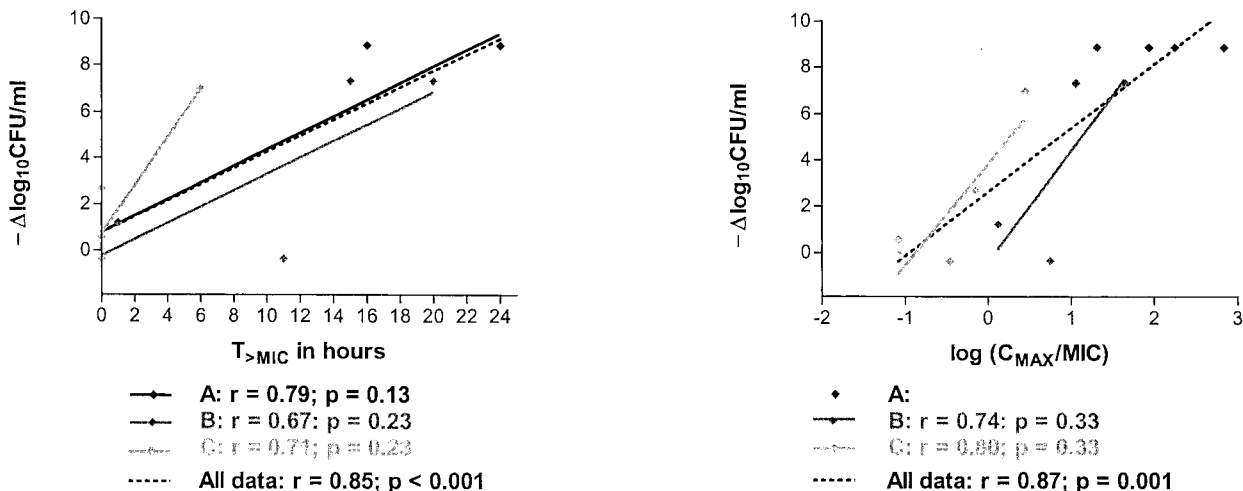

FIG. 6. Correlations between efficacy and $T_{>MIC}$ and between efficacy and the C_{max}/MIC ratio in the rabbit model. y axes show the differences in numbers of CFU between treated animals and untreated controls.

Using different models to correlate the PK and PD parameters with efficacy awakes the discussion on different end points and especially the time used as the end point. In both the mouse models, 6 h was used since the bacteria showed growth for at least this period, penicillin has a short elimination $t_{1/2}$ in serum in mice (12 min), and the treatment regimens had to be extended to this period. In the rabbit tissue cage model, penicillin had a longer $t_{1/2}$ (3 h), the bacterial exponential growth phase was approximately 9 h, and the intended treatments with various PK and PD parameters could be given in one dose. The times chosen for the end points can be crucial; e.g., under circumstances in which no regrowth or changes are seen after an initial killing effect, a doubling of the observation time would reduce the $T_{>MIC}$ by half if the value is expressed as a percentage but not if it is expressed as an exact figure such as number of minutes or hours. When efficacies in different models are compared, it seems more obvious to use absolute figures rather than percentages. When the data from the rabbit model were analyzed using the different end points of 12 and 24 h, the same correlations with $T_{>MIC}$ were found, and a $T_{>MIC}$ of approximately 50% was necessary to achieve maximal killing in the model. For the C_{max}/MIC ratio, the threshold was approximately 10 under both conditions. Since changing the times used as end points did not change the conclusions, the end point of 24 h was chosen for comparison with previous results with this model (7).

Only minor differences in the behaviors of the strains were recognized; in both mouse models, the growth of strain B was somehow impaired in the mixed-culture infections, and this impairment was also seen in vitro but not in the rabbit tissue cage model with the mixed cultures and not in experiments with the mouse peritonitis model in which the strain was inoculated separately. This difference in the growth abilities of strain B is unexplained.

It was possible to perform insufficient treatments, selecting for resistant bacteria in all the models, as intended. In the rabbit tissue cage model where the observation time was 24 h, it was possible to observe the regrowth of the resistant bacteria, initially suppressed by the treatment but obviously not eradicated. In the mouse models, the selective pressure of the treatments was seen, with selection of the less-susceptible strains in the peritonitis model and decreases in the relative amounts of the susceptible strains during treatments in the thigh model.

The main result of this study was the finding that the eradication of the bacteria, independent of the susceptibility of the strain or strains, followed the well-known PK and PD rules for treatment with β-lactams regardless of whether the strains were used in a mixture or separately. Maximal efficacy was seen when the $T_{>MIC}$ was >40 to 50% of the observation time and the C_{max}/MIC ratio was >10. The importance of both the parameters, $T_{>MIC}$ and C_{max}/MIC, has been described in a previous study comparing the efficacies of penicillin in four different animal models with the same pneumococcal strains (7). Also, with the use of an in vitro kinetic model, it has been shown that a $T_{>MIC}$ of 50% is required to obtain maximal efficacy of amoxicillin against a penicillin-susceptible and a penicillin-intermediate (MIC = 0.25 μg/ml) strain of *S. pneumoniae*. However, for a strain for which the MIC was 2 μg/ml, a $T_{>MIC}$ of 60% and a C_{max} of 10 times the MIC were needed (11).

The finding that the C_{max}/MIC ratio should be above 10 in β-lactam regimens will be clinically unimportant in many cases, since most β-lactams have short $t_{1/2}$s; e.g., the $t_{1/2}$ of penicillin G in humans is approximately 30 min (8). Therefore, only seldom will the C_{max}/MIC ratio be below 10 when the $T_{>MIC}$ is above 40%, as widely recommended (5). If a low dose is used for continuous infusion, there is a risk of this ratio being below 10, which would not be recommendable in view of our data.

In this study, we used three strains of pneumococci for which the MICs were different but the serotype was the same in order to minimize factors other than the PK and PD parameters influencing the possible selection. In clinical settings, the potential pathogens are probably often of different species and have different levels of virulence. Thus, a selective treatment may be selective not only of less-susceptible subpopulations within the same species of bacteria, such as different clones of pneumococci, but also of different species colonizing the same area, such as *Haemophilus* spp., *Moraxella catarrhalis*, and various other *Streptococcus* spp. colonizing, e.g., the nasopharynxes of infants or middle-ear fluids of children (6, 16).

The selective pressure in clinical settings was shown by Guillemot et al. (10), who demonstrated that low-dose treatments with β-lactams over a longer period (>5 days) increase the risk in children of being colonized with resistant pneumococci (10). The importance of eradicating the possible pathogens when antibiotics are used has recently been highlighted in a review of otitis media and respiratory tract infections (6). Eradication is essential not only for curing the patient and diminishing the risk of treatment failure but also for minimizing the risk of resistance developments in the pathogenic bacteria (6).

In conclusion, it was possible to induce the selection of resistant strains by the use of suboptimal dosing regimens in the three different animal models used in this study. Efficacy was found to be significantly correlated with both $T_{>MIC}$ and the C_{max}/MIC ratio when data for all strains were collected in all models. These findings confirm the importance of eradicating all the bacteria taking part in the infectious process in order to avoid selecting for resistant subpopulations or clones.

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