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Experimental *Streptococcus pneumoniae* Infection in Mice for Studying Correlation of In Vitro and In Vivo Activities of Penicillin against Pneumococci with Various Susceptibilities to Penicillin

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The purpose of the study was to investigate the correlation of in vitro activity with the in vivo effect and the pharmacokinetics of penicillin in the treatment of infections with pneumococci with various susceptibilities to penicillin. We used 10 pneumococcal strains for which penicillin MICs ranged from 0.016 to 8 μg/ml. Time-kill curve experiments were performed with all strains. We found that the effect of penicillin in vitro is concentration independent, with a maximum effect at two to four times the MIC for penicillin-susceptible as well as penicillin-resistant pneumococci. The mouse peritonitis model with an inoculum of approximately 10^6 CFU, to which mucin was added, resulted in a reproducible lethal infection with the pneumococci. The 50% effective dose was determined for each strain, and we found a highly significant correlation between the log MIC and the log 50% effective dose of penicillin against these strains ($P < 0.01$). Furthermore, it was shown that the most important pharmacokinetic parameter determining the effect of penicillin in vivo was the time that the concentration of penicillin in serum was greater than the MIC.

Despite the developments in antibiotics and vaccines, *Streptococcus pneumoniae* is still an important cause of pneumonia and meningitis (7). The increasing resistance of pneumococcal strains to penicillin in recent years and the spread of these resistant strains to many parts of the world have renewed interest in treatments for pneumococcal infections (1, 6, 22, 27). Penicillin resistance is often followed by resistance to other antibiotics, e.g., chloramphenicol, macrolides, sulfonamides, and tetracyclines (17, 22). Pneumococci are classified as fully susceptible to penicillin when the MIC is less than 0.1 μg/ml, intermediately resistant when the MICs are at or between 0.1 and 1 μg/ml, and highly resistant when the MIC is greater than 1 μg/ml (22). In some cases of infection, higher doses of penicillin have had a sufficient effect; otherwise, other beta-lactam antibiotics such as the broad-spectrum cephalosporins and glycopeptides are recommended for use in the treatment of infections caused by these pathogens (1, 6, 7, 17, 22).

We have previously shown in an experimental animal model that the most important pharmacokinetic parameter indicating an effect of beta-lactam antibiotics in vivo against penicillin-suspectible pneumococci is the time that the antibiotic concentration remains greater than the MIC ($T_{>MIC}$) (12, 13). Whether the same pharmacodynamic rules count for penicillin-resistant pneumococci has been the subject of only a few experimental studies, and then with only a few strains (2, 5, 16). The purpose of the present study was to investigate the correlation of in vitro activity with the in vivo effect and the pharmacokinetics of penicillin in the treatment of infections with pneumococci with various susceptibilities to penicillin. We used 10 pneumococcal strains for which penicillin MICs ranged from 0.016 to 8 μg/ml in the mouse peritonitis model.

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MATERIALS AND METHODS

Bacteria, media, and growth conditions. Ten clinical isolates of pneumococci from different parts of the world were used. They were selected to represent a spectrum of strains for which penicillin MICs ranged from 0.016 to 8 μg/ml. Serotype 6A, 6B, 14, and 19A strains were used (Table 1). The strains were kindly provided by K. Kristinsson, Department of Microbiology, National University Hospital, Reykjavik, Iceland, and J. Henrichsen, Statens Seruminstitut, Copenhagen, Denmark. The serotypes were determined at The Streptococcus Department, Statens Seruminstitut, by using anti-capsular polysaccharide antibodies (24).

Bacterial suspensions for inoculation were prepared from fresh overnight cultures on 5% blood agar plates made from frozen stock cultures. The inoculum was prepared immediately before inoculation by suspending the colonies in sterile beef broth medium (Statens Seruminstitut) and was adjusted to an optical density at 540 nm of 0.5, giving a density of approximately 10^8 CFU/ml. For each experiment, the size of the inoculum was determined after making 10-fold dilutions in beef broth; this was followed by plating 0.1 ml on 5% blood agar plates, with subsequent counting of the colonies.

Beef broth, 5% blood agar plates, Mueller-Hinton plates supplemented with 5% lysed horse blood, and Danish blood agar plates (19) were produced at the Statens Seruminstitut. Mucin (M-2378; Sigma Chemical Company, St. Louis, Mo.), an enzyme extract of porcine stomach, was used as adjuvant for inoculation of the mice and was prepared as a saline stock solution of 10% (wt/vol). Immediately before inoculation, the mucin solutions were diluted 1:1 with pneumococcal suspensions, giving a final mucin concentration of 5% (wt/vol). Mucin was diluted in beef broth to 5% (wt/vol) for time-kill experiments.

MICs, MBCs, and time-kill curves. The MICs were determined by four different methods: (i) broth macrodilution in glass tubes, (ii) broth dilution in microtiter plates (Nunc Microwell Plates 262170; A/S Nunc, Roskilde, Denmark), (iii) the plate dilution method, and (iv) the E test (E test; AB Biodisk, Solne, Sweden). The E test is performed as a disk diffusion method with a strip with gradient concentrations of an antibiotic. All tests were done in duplicate, and the results were read after 20 h of incubation at 35°C. Beef broth to which 5% sheep blood was added was used for the broth dilution methods, in which tubes and microtiter plates with inocula of 10^5 to 10^6 CFU/ml were used. Penicillin G (Penicillin; Leo Pharmaceuticals, Ballerup, Denmark) was diluted twofold in beef broth at concentrations of 0.003 to 64 μg/ml. The MIC was determined to be the lowest concentration of antibiotic in which there was no visible growth. The MBC was determined by the microtiter broth dilution method only by plating 100 μl from each well without visible growth on 5% blood agar plates containing β-lactamase (Penase; Leo Pharmaceuticals), and the MBC was de-

TABLE 1. Serotypes, origins, and MICs and MBCs of penicillin for 10 pneumococcal strains measured by different methods

Strain no.; country of origin	Serotype	MIC (μg/ml)		Microtiter MIC/MBC (μg/ml)[a]	E test MIC (μg/ml)[c]
		Broth dilution[a]	Plate dilution[b]		
68128; ND[d]	14	8	16	32/32	16
2916; Czechoslovakia	14	4	16	8/8	6
1320; Iceland	6B	2	4	4/4	1
1189; Iceland	6B	1	2	2/2	0.25
625; Hungary	6B	1	2	2/2	0.75
902; Iceland	6B	1	2	1/1	0.5
999; Iceland	19A	0.5	0.25	0.25/0.25	0.25
1064; Iceland	6B	0.25	0.25	0.25/0.25	0.125
L; United States	6A	0.016	0.25	<0.06	0.012
493/73 Denmark	6B	0.016	0.015	<0.06	0.008

[a] Beef broth with 5% sheep blood.
[b] Mueller-Hinton agar plates with 5% lysed horse blood.
[c] Danish blood agar plates (Statens Seruminstitut).
[d] ND, not determined.

fined as the lowest concentration that reduced the CFU by 99.9% or more. Mueller-Hinton agar plates supplemented with 5% lysed horse blood were used for plate dilution MIC determinations. The plates contained penicillin G at concentrations of 0.003 to 128 μg/ml. The inoculum, 10^4 CFU per spot (10^7 CFU/ml), was applied to the plates with a multipoint inoculator (A400; Denley Instruments Ltd., Sussex, United Kingdom). The MIC was defined as the lowest concentration of penicillin G at which one or no colonies were seen. The E test was performed on Danish blood agar plates, and the MIC was determined according to the manufacturer's instructions.

Time-kill curves for penicillin were performed with concentrations of 0, 0.5, 1, 2, and 4 times the MICs for each pneumococcus in flasks containing 20 ml of beef broth with an inoculum of 10^6 CFU/ml in a shake stand. After 30 min of incubation at 35°C, samples of 1 ml were drawn and were replaced by penicillin solutions containing 20 times the final concentration of pencillin or, for the antibiotic-free control, pure beef broth. Samples were then taken after 3 and 6 h of incubation. The numbers of CFU per milliliter were determined after making appropriate dilutions, and 0.1 ml was spread onto 5% blood agar plates. Colony counts were performed after 20 h of incubation at 35°C. All time-kill curve experiments were performed in quadruplicate.

Mouse peritonitis model. Outbred, female *ssc* CF-1 mice (ages, 8 to 12 weeks; weight, 30 ± 2 g) were used throughout the study. The mice were kept in cages at five mice to a cage, and they had free access to chow and water. Inoculation was performed by intraperitoneal injection of an inoculum of 0.5 ml of pneumococcal suspension via a 25-gauge syringe. Blood samples were obtained through periorbital cuts after anesthetizing the mice with CO_2. After sacrificing the mice, peritoneal washes were performed by injecting 2 ml of sterile saline intraperitoneally and then massaging the abdomen and opening the peritoneum to collect the fluid (14). Blood and peritoneal fluid samples were immediately diluted, and 0.1 ml was plated onto 5% blood agar plates. Treatment of mice with single doses of penicillin was performed by administering subcutaneous injections in the neck region at a volume of 0.5 ml per dose.

Determination of LD_{100} for individual pneumococci with or without mucin. A dose giving 100% lethality (LD_{100}), but not necessarily the lowest dose giving 100% lethality, of each pneumococcal strain was determined by performing experiments with inocula of 10^2, 10^4, 10^6, and 10^8 CFU/ml, with or without the addition of 5% (wt/vol) mucin; each inoculum of each strain was given to two mice. The mice were observed for 5 days. The potential lethal effect of mucin alone was examined by intraperitoneally inoculating 0.5 ml of 5 or 10% (wt/vol) mucin-saline solutions to each mouse. None of these mice died within the 5 days of observation.

In vivo growth curves. The in vivo growth curves were determined by inoculating mice in groups of nine mice each with 10^6 to 10^8 CFU/ml, the former with or without 5% (wt/vol) mucin. Blood and peritoneal fluid were obtained from three mice each after 1, 3, and 6 h.

Determination of the ED_{50} of penicillin for individual pneumococci. The 50% effective doses (ED_{50}s) of penicillin for the individual strains were determined in two or three experiments, with a minimum of 50 mice infected with each bacterial strain receiving the same inoculum of 1×10^6 to 10×10^6 CFU/ml with 5% mucin. Penicillin was administered 90 min after inoculation, and the mice were observed for 5 days. At each dosage, penicillin was given to a minimum of five mice each infected with each pneumococcal strain. If the ED_{50} could be calculated from the first trial of 25 mice infected with each strain, the trial was repeated with the same dosages. The method of Reed and Muench (29) as well as the Hill equation was used to calculate the ED_{50} (InPlot; Scientific Graphics, GraphPad Software, San Diego, Calif.). If the ED_{50} was not reliably determined in the first trial, dosages were adjusted in the second trial again by using 25 mice infected with each strain and the ED_{50} was confirmed in a third trial.

Determination of penicillin concentrations in serum. The cup plate bioassay method was used to measure the penicillin concentration in mouse serum. A nonhemolytic *Streptococcus* strain (strain EB-68, Department of Antibiotics, Statens Seruminstitut) was used for the bioassay. After collection of blood samples, the blood was centrifuged at $1{,}630 \times g$ for 10 min, and the serum was stored at −80°C until analysis, which was performed in duplicate. For standard curves, penicillin was diluted in pooled normal mouse serum. Studies of the pharmacokinetics of penicillin in mice were performed in experiments with treated animals in groups of three each. Penicillin was administered by subcutaneous injection as described above, and the mice were bled after anesthesia with CO_2. Serum samples were obtained after 10, 20, 30, 40, and 60 min for a range of penicillin dosages. Values in the interval between 0.2 and 0.01 μg/ml were also extrapolated, since the lowest detectable value in the bioassay was 0.2 μg/ml. The ED_{50}s were used and were determined as described by Reed and Muench (29) (see above). The highest concentration measured in serum (C_{max}) was, in all cases, at 10 min. The serum elimination half-life was estimated by the expression $-\log 2/\beta$, where β is the slope of the serum elimination regression line (log serum concentration versus time). $T_{>MIC}$ was estimated from the serum elimination regression line. The area under the serum concentration curve (AUC) was calculated by the trapezoidal rule.

Statistical methods. Besides the method of Reed and Muench (29), we also used the Hill equation to calculate the ED_{50} with 95% confidence limits, the maximum ED_{50} (E_{max}), the minimum ED_{50} (E_{min}), and the slope of the dose-effect curve with 95% confidence limits. The Spearman's rank correlation test was used for correlation analyses. The Sign's test was used to evaluate the effect of mucin on pneumococcal lethality. $P < 0.05$ was considered significant.

RESULTS

MICs, MBCs, and time-kill studies in vitro. The MICs, the MBCs, and serotypes of the 10 pneumococci are given in Table 1. The MICs determined by the plate dilution method and in microtiter plates were, for most strains, one twofold dilution

TABLE 2. Time-kill experiments

Penicillin concn (fold MIC)	Time (h)	Difference in log CFU/ml[a]
0.5	3	0.7 (0.3–1.3)
	6	2.4 (0.4–3.5)
1	3	1.5 (0.8–2.6)
	6	3.1 (1.5–4.6)
2	3	2.1 (1.5–2.9)
	6	3.7 (2.7–4.2)
4	3	2.2 (1.8–2.6)
	6	3.9 (3.0–5.0)

[a] Differences between control- and penicillin-containing flasks for 10 pneumococci at various concentrations of penicillin. Values are median (range).

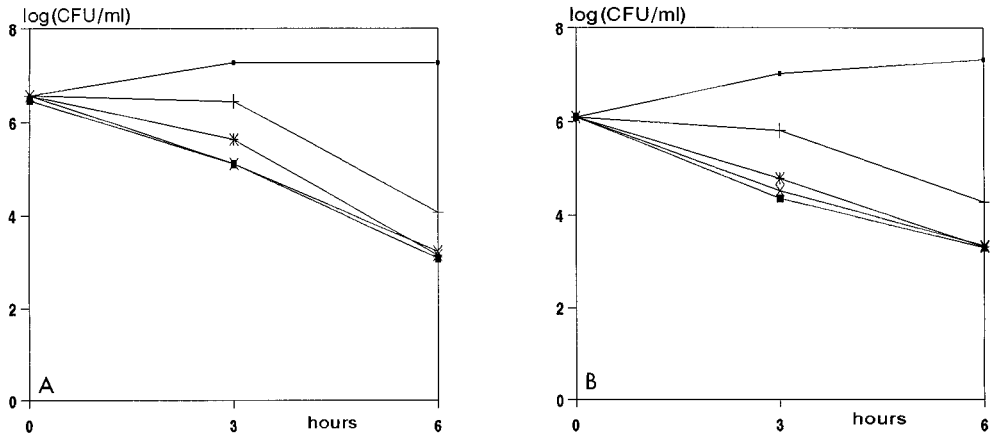

FIG. 1. Examples of time-kill curves for pneumococci and penicillin. (A) Strain 68128; penicillin MIC, 8 μg/ml. (B) Strain 493/73; penicillin MIC, 0.016 μg/ml. ■, control; +, 0.5 × MIC; ∗ (top), 1× MIC; ■, 2× MIC; ∗ (bottom), 4× MIC.

step greater than the results obtained by the broth dilution method, while the results of the E test were, for most strains, less than one twofold dilution step from the broth dilution MICs that were obtained.

The data from the time-kill curves, expressed as the differences in the log CFU per milliliter between control- and penicillin-containing flasks with different concentrations of penicillin after 3 and 6 h, are given in Table 2, and examples are given in Fig. 1. The killing effect increased both with increasing concentrations of penicillin and with time, but the effect of killing after 3 and 6 h did not show any correlation with the actual MIC for the 10 strains (Spearman's rho for the 10 strains was a median of −0.40 [range, −0.58 to −0.07; $p > 0.05$]). Time-kill curves for strains 68128 and 2916 were also performed by using concentrations of penicillin 8 and 16 times the MIC, respectively, but no additional effect compared with that at 4 times the MIC was observed (data not shown). For strain 68128, the time-kill curve was done with the addition of 5% mucin, but no difference compared with the control without mucin was observed (data not shown).

Intraperitoneal infection with or without the addition of mucin. In order to secure the effect of penicillin in vivo, it was important to prove that the pneumococci are in an exponential growth phase at the time of penicillin administration. Figure 2 shows that the addition of mucin to the inoculum resulted in an exponential growth curve in vivo in both blood and peritoneal fluid; this exponential growth curve could not be identified in the peritoneal fluid when the higher inoculum was used. The addition of mucin increased the virulence of all strains. The median (range) LD_{100}s were 8.5 log CFU/ml (7.9 to 8.7 log CFU/ml) and 4.4 log CFU/ml (2.4 to 7.1 log CFU/ml) without and with mucin, respectively; i.e., the mucin significantly lowered the LD_{100}.

Determination of the ED_{50} of penicillin for the 10 pneumococcal strains in mice. Table 3 provides the ED_{50}s determined by both the method of Reed and Muench and the Hill equation (an example is shown in Fig. 3). The 95% confidence interval obtained for the ED_{50}s determined by the Hill equation indicated a low variation except for that for strain 68128. Also, the two methods of determining the ED_{50}s gave nearly identical results for nine of the strains (Spearman's rho = 0.98; $P < 0.01$) (Table 3). For one strain, strain 1320, it was not possible to obtain estimates by the Hill equation since only one value between E_{max} and E_{min} could be reliably obtained. The log MIC for the pneumococcal strains showed a highly significant correlation with both log ED_{50}s (Spearman's rho = 0.96; $P < 0.01$; $n = 10$ [by the method of Reed and Muench (29)]; Spearman's rho = 0.95; $P < 0.01$; $n = 9$ [by the Hill equation]). Figure 4 shows the relationship between the log MIC and the log ED_{50} (as determined by the method of Reed and Muench [29]).

Pharmacokinetic studies of penicillin G in mice after subcutaneous injections. Pharmacokinetic studies of penicillin G given subcutaneously to mice showed a median serum elimination half-life of 7.7 min (range, 7.2 to 9.7 min) at dosages of between 1 and 66 μg/mg of body weight. The results of the pharmacokinetic studies according to the dosages used are given in Table 4. $T_{>MIC}$ was the most constant parameter, as indicated by the most narrow range (24 to 60 min); this followed by C_{max}/MIC, with a range of from 9 to 96 (Table 4). The other parameters such as AUC (range, 23 to 3,500 μg · min/ml), C_{max} (range, 0.8 to 70 μg/ml), and the AUC/MIC ratio (range, 438 to 4,300 min) showed a much higher variation (Table 4).

Pharmacokinetic parameters of penicillin G and in vivo effect compared with in vitro effect against pneumococci in mice. Because the log MIC correlated with the log ED_{50}, the

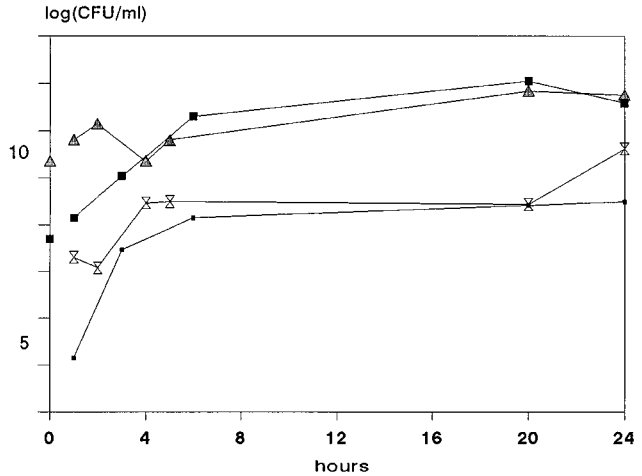

FIG. 2. Growth curves in vivo for strain 68128. Inoculum of 0.5 ml of 10^6 CFU/ml and 5% mucin (■, blood; ■, peritoneal fluid). Inoculum of 0.5 ml of 10^8 CFU/ml (⧖, blood; △, peritoneal fluid).

TABLE 3. Effect of penicillin against 10 pneumococci in the mouse peritonitis model[a]

Strain (no. of mice)	MIC (μg/ml)	Reed and Muench ED_{50} (mg/kg)	Hill equation				
			ED_{50} (95% CI[b])	E_{max} (mg/kg)	E_{min} (mg/kg)	Slope[c] (95% CI)	E_{max}/E_{min}
68128 (60)	8	42.32	45.09 (10.59–91.82)	84.11	14.39	3.64 (−15.80–23.07)	5.8
2916 (60)	4	45.85	47.78 (29.21–78.18)	246.31	13.17	2.08 (0.01–4.17)	18.9
1320 (65)	2	35.67	—[d]	—	—	—	—
1189 (50)	1	19.73	20.37 (18.82–22.64)	103.03	3.80	2.73 (2.20–3.28)	27.1
625 (50)	1	20.81	20.03 (17.75–22.60)	144.15	6.70	3.66 (2.28–5.03)	21.5
902 (60)	1	21.18	21.78 (21.21–22.35)	56.67	5.48	5.84 (5.19–6.49)	8.5
999 (65)	0.5	2.79	2.70 (2.41–3.03)	11.89	1.44	6.07 (2.08–10.06)	8.3
1064 (60)	0.25	9.85	11.18 (9.43–13.26)	72.81	1.02	1.86 (1.22–2.50)	71.4
L (74)	0.016	2.00	1.92 (1.48–2.48)	10.75	0.33	2.69 (0.91–4.46)	3.6
493/73 (75)	0.016	0.76	0.67 (0.44–1.00)	14.54	0.36	1.27 (0.93–1.61)	40.4

[a] The ED_{50} was calculated as described by Reed and Muench (29), and ED_{50}, E_{max} and E_{min}, the ratio of E_{max}/E_{min}, and the slope were calculated by the Hill equation (11).
[b] CI, confidence interval.
[c] Percentage of surviving animal per milligram of penicillin.
[d] —, not done; see text.

log MIC was correlated with log E_{max} and log E_{min} (Spearman's rho = 0.75 and $P = 0.02$ for E_{max}; Spearman's rho = 0.98 and $P < 0.01$ for E_{min}).

The slopes of the curves obtained by the Hill equation showed great variation, as also indicated by the 95% confidence limits in Table 3. Neither the slope nor the ratio of E_{max}/E_{min} showed any correlation to the log MIC for the nine strains (Spearman's rho = −0.19 and $P > 0.05$ for slope; Spearman's rho = 0.396 and $P > 0.05$ for E_{max}/E_{min}). When comparing the degree of the killing effect of penicillin in vitro and in vivo, the only correlation besides that between MIC and ED_{50} was found between the slope of the curves obtained by the Hill equation and the killing rate after 6 h at two and four times the MICs (Spearman rho = −0.73 and $P = 0.03$ for two times the MIC; Spearman's rho = −0.69 and $P = 0.04$ for four times the MIC).

DISCUSSION

In previous studies from this laboratory, the mouse peritonitis model was used with a penicillin-susceptible pneumococcus serotype 3 strain in order to obtain a reproducible experimental model in which the infection induced by a low inoculum was lethal to mice with normal immune function (12–14). Within this context it was possible to show that the most important pharmacokinetic parameter for determining the in vivo effect of beta-lactam antibiotics, as exemplified by various penicillins and cephalosporins, was the $T_{>MIC}$ (12, 13, 15). The results of those studies were later confirmed either in a similar mouse peritonitis model with different pathogens (33, 34) or in the neutropenic mouse thigh model (23, 35). Original studies by Eagle and Musselmann (9) demonstrated that penicillin has time-dependent and concentration-independent effects.

The concept of studying the pharmacodynamics, i.e., the correlation of the effect in vivo with the pharmacokinetics, in experimental models with several different bacteria with various susceptibilities to the antibiotics in question has been attempted by several investigators (2, 5, 11, 16, 28, 32, 35). If different bacteria are used, the question arises as to whether the infections caused by bacteria with different virulence properties and, possibly, the different immune responses elicited by the host can provide any meaningful insight into the pharmacodynamic properties of different antibiotics with different modes of action (2, 11, 35).

It would therefore be preferable to use bacteria of the same genus with variable but stable susceptibilities to the study drugs. Penicillin-resistant pneumococci were therefore obvious choices for the present study. The mechanism of penicillin resistance, i.e., changes in penicillin-binding proteins, in these

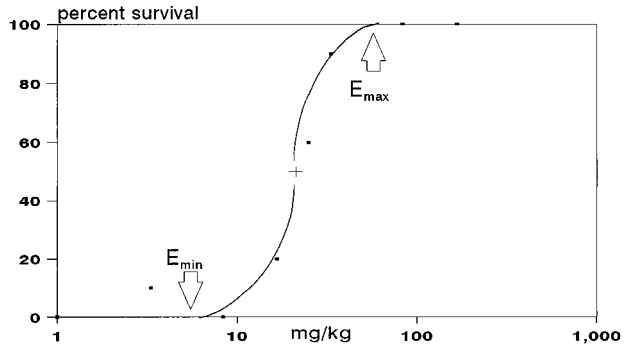

FIG. 3. Example of ED_{50} determination showing results for strain 902. E_{max} and E_{min} are indicated by arrows. The calculated ED_{50} is marked with a plus sign.

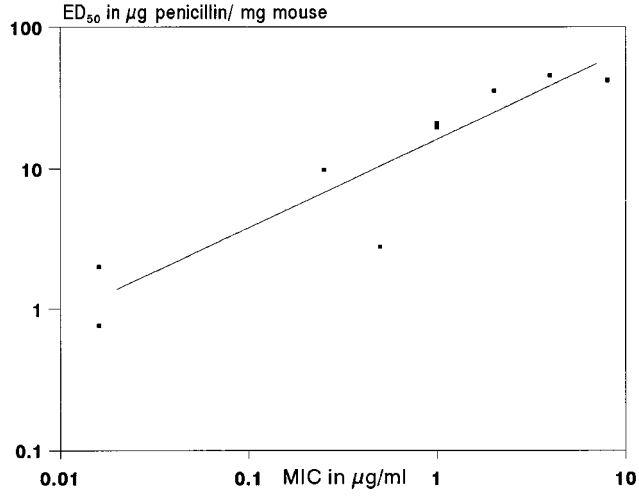

FIG. 4. Log ED_{50} versus log MIC for 10 pneumococci (the (ED_{50} was calculated as described by Reed and Muench [29]).

TABLE 4. Pharmacokinetics of penicillin in mice determined at the ED_{50}s for 10 pneumococci

Strain	$T_{>MIC}$ (min)	C_{max} (μg/ml)	AUC (μg · min/ml)	C_{max}/MIC	AUC/MIC (min)
68128	24	70	3,500	9	438
2916	32	70	3,500	18	875
1320	36	47	2,265	24	1,133
1189	42	38	1,794	38	1,794
625	42	38	1,794	38	1,794
902	42	38	1,794	38	1,794
999	31	8	316	16	632
1064	60	24	1,075	96	4,300
L	58	1.5	49	94	3,063
473/93	50	0.8	23	50	1,438
Median (range)	42 (24–60)	38 (0.8–70)	1,794 (23–3,500)	38 (9–96)	1,616 (438–4,300)

bacteria is relatively stable (i.e., it is not lost during storage or during the infection process) and noninducible (i.e., it does not depend on degrading enzymes) (22). Furthermore, susceptible strains do not develop penicillin resistance during treatment.

The MICs vary according to the methods used (Table 1) (4), but apparently, the variations are constant at all MICs. For further analysis we settled for the broth macro dilution method for determination of MICs because it was the standard method in our laboratory. The E test seems to be reliable for determination of the MICs for pneumococci (18, 20, 25), and we also found acceptable concordance of the E test result with those of the methods used to test these 10 strains. The effect of penicillin in time-kill studies showed relatively little variation among the strains and confirmed the results of previous studies, that is, that the maximal killing effect of penicillin against pneumococci was in the range of two to four times the MIC, irrespective of the MIC for the strain (Table 2) (31).

The virulence of pneumococci to mice varies according to capsular type (26). Interestingly, penicillin-resistant pneumococci are dominated by capsular types with low levels of virulence to mice, i.e., types 14, 19, and 23F (26). This was confirmed in the present study, in which the LD_{100}s were considerably higher for these strains than for serotype 3 strains (15). All strains of type 6B showed low-level virulence properties in the present study, although Mørch (26) ranked them as highly virulent. The addition of mucin to the inoculum considerably reduced the LD_{100} for all strains. Mucin blocks the peritoneal macrophage function at least temporarily and thereby facilitates the spread of the bacteria from the peritoneal cavity (30). A similar effect of enhancing the virulence of staphylococci for mice is seen when adding a range of high-molecular-weight human blood proteins (10, 21). A lower inoculum appeared to be important in order to show an exponential growth curve in vivo (Fig. 2), which is necessary for penicillin treatment to have an effect in vivo (14). For determination of the ED_{50}s of penicillin, the same inoculum of all strains of 0.5 ml of 1×10^6 to 5×10^6 CFU/ml was used. With respect to the ED_{50}s, the slope of the dose-effect curve as calculated by the Hill equation (Table 3) indicates the range of doses needed for determination of the ED_{50}; the slopes varied considerably among the nine strains for which it could be calculated, and this variation was also indicated by the ratio between E_{max} and E_{min}. The slopes showed no correlation with MICs for the strains tested, which corresponds to the concept that penicillin shows a concentration-independent killing effect. A borderline significant inverse correlation between slopes and killing effect was found. This was measured by a reduction in the colony counts after 6 h at two and four times the MIC, but the correlation coefficients signified that only about 50% ($rho^2 = 53$ and 48%, respectively) of the variation in the slopes could be explained by the variation in killing rates. Whether the variation in the slopes is due to differences in virulence among the strains, differences in the action of penicillin on the strains in vivo, or other factors cannot be deduced from our results.

In spite of the variation in slopes, there was a highly significant correlation between log MIC and log ED_{50} (Table 3; Fig. 4), as well as between log MIC and log E_{max} and log E_{min}. A similar high degree of correlation between log MIC and log ED_{50} was shown previously for 14 different cephalosporins in the same model with a penicillin-susceptible serotype 3 strain (12). The relationship indicates that the MIC is highly predictive of the dose of penicillin needed for the drug to have an effect. When further incorporating the pharmacokinetic parameters (Table 4), it was evident that the most important parameter for determination of an effect in vivo is $T_{>MIC}$; when comparing the different pharmacokinetic parameters determined at the ED_{50}s for each strain, the $T_{>MIC}$ varied only by a factor of less than 2.5 between the highest and lowest value, in contrast to factors from 10 to greater than 100 for the other parameters tested (Table 4). This result confirms the results of the previous studies with the penicillin-susceptible strain (12) and follows the general rule that for antibiotics such as beta-lactam antibiotics that show concentration-independent killing effects in vitro, $T_{>MIC}$ is the most important factor for determining the outcome of infection (3, 8). The effects of higher doses of amoxicillin when a penicillin-resistant pneumococcus instead of a penicillin-susceptible strain was used was shown in an otitis media model in gerbils (5). Clinical experience has already suggested that increasing the penicillin dose is sufficient for this drug to have an effect against infections with penicillin-resistant pneumococci, as long as the dose can be tolerated by the patient (17, 22). Dosing regimens which secure penicillin concentrations above the MIC for the infecting bacteria for an extended period of time, i.e., frequent dosing, constant infusion, or the concomitant use of probenecid, could then be suggested as the most effective way of administering this drug for the treatment of infections caused by penicillin-resistant pneumococci.

In conclusion, the present study showed that the killing effect of penicillin in vitro is concentration independent, with a maximum at two to four times the MIC for penicillin-susceptible as well as penicillin-resistant pneumococci. A mouse peritonitis model in which mucin was added to the inoculum resulting in a reproducible lethal infection with pneumococci with various susceptibilities to penicillin was used to show that there is a highly significant correlation between the log MIC and the log ED_{50} of penicillin for these strains. Furthermore,

and as demonstrated previously with a single strain with a range of beta-lactam antibiotics in the same model, it was shown that the most important pharmacokinetic parameter for determining the effect of penicillin in vivo is $T_{>MIC}$.

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