Reverse Inoculum Effect in Bactericidal Activity and Other Variables Affecting Killing of Group B Streptococci by Penicillin

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Variables of the effect of penicillin G on the numbers of viable group B streptococci in broth cultures were studied. One-fourth of the MIC was the lowest concentration that reduced the viable count compared with antibiotic-free controls. The rate of killing increased with the concentration of penicillin up to 4× MIC, but no further. During the first 2 or 3 h, the bactericidal activity was more rapid than later on. The MIC and supraoptimal concentrations of penicillin killed an inoculum of 10⁶ organisms more rapidly than an inoculum of 10⁴ organisms. The MIC was not inoculum dependent. The reverse inoculum effect was revealed by the killing curves but not by the MBC. There were reproducible differences among strains as to the rate of killing by penicillin; these did not correlate with the rate of multiplication, which also varied among strains. Among the 11 strains tested, there were no tolerant ones.

Huge numbers of antimicrobial susceptibility tests are performed daily to predict therapeutic efficacy. Such predictive value is known in only few instances (10). Most tests detect growth inhibitory activity, and dissociation of in vitro and in vivo results is to be expected when bactericidal activity is needed for cure, as is believed to be the case with infections of immunocompromised patients or with infections, like endocarditis, of immunologically privileged sites. Group B streptococci are susceptible to penicillin in the conventional sense, but neonatal infections have turned out to be difficult to treat, and failures have been ascribed to penicillin tolerance, i.e., relative resistance to the bactericidal effect (4, 23).

The description of bactericidal activity has remained a problem. The most widely used parameter is the MBC, and we have characterized several variables of the effect of penicillin on the numbers of viable group B streptococci to see to what extent the MBC reveals all relevant information about bactericidal activity. The model combination was chosen, because it has been studied extensively before, because it is one of the combinations with which phenomena like paradoxically reduced killing at high antibiotic concentrations (7) and antibiotic tolerance have been described, and because it has been associated with therapeutic failures.

MATERIALS AND METHODS

Bacteria. Group B streptococci were isolated from clinical specimens from various male and female genital, throat, nose, ear, and skin infections as the predominant probable pathogen. They were identified by three presumptive tests, hippurate hydrolysis, cAMP reaction, and pigment production, as well as serologically, as described earlier (14). Thirty-five isolates were selected randomly for these studies from a collection maintained lyophilized and by serial passage on blood agar plates.

Inoculum. In preliminary studies of 18- to 21-h growth curves, supplementing Todd-Hewitt broth (BBL Microbiology Systems, Cockeysville, Md.) with 0.8% glucose reduced the growth of group B streptococci, and 10% horse serum had essentially no effect. Thus, plain Todd-Hewitt broth was

chosen as the medium. Overnight growth in broth was diluted in fresh broth to ca. 10 CFU/ml and incubated in 1-ml volumes at 36°C. Samples were taken at 3-h intervals, and duplicate 0.1-ml samples of appropriate dilutions (1:10 steps mixed by vigorous shaking) in saline were plated on 9-cm blood agar plates for colony counting after overnight incubation. The growth curves of nine group B streptococci were so determined (Fig. 1). Similar cultures were subsequently used to prepare predictable inocula for the experiments: exponential-phase bacteria were obtained after 9 h of incubation. The size of each inoculum was also individually determined by colony counting.

Determination of MIC and killing. Penicillin G (Farbwerke Hoechst AG, Frankfurt/Main, Federal Republic of Germany) was dissolved in sterile water to 1 mg/ml and kept at -20°C. Appropriate dilutions of the stock solution were prepared in 0.5-ml volumes of broth before each experiment, equal volumes of the inoculum suspension were added, and the mixtures were incubated at 36°C. The MIC was the lowest concentration in a series of twofold drug dilutions which allowed no visible growth after overnight incubation. Samples of 0.05 or 0.1 ml for dilution and colony counting were taken from similar 1-ml cultures to determine drug effects on the numbers of viable bacteria. In all killing experiments each duration of exposure to penicillin was represented by a tube of its own, and each tube was visited only once and then discarded. The effect of carry-over penicillin on colony counts was eliminated by dilution and spreading on agar.

Generation times. In the standard 1-ml broth cultures, partial growth curves of 32 group B streptococci were determined to screen for rapid and slow growers. Samples for colony counting were taken after incubation for 2 and 6 h, and the generation time (T_2) was calculated as follows: $T_2 = 4 \text{ h} \times \log_2/(\log C_6 - \log C_2)$, where C_2 is the colony count in CFU/ml at 2 h and C_6 is that at 6 h.

RESULTS

Effect of penicillin concentration. The effects of time and penicillin concentration on the numbers of viable group B streptococci in the present experimental system were described with four bacteria, whose simultaneously deter-

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FIG. 1. Growth curves in Todd-Hewitt broth of nine group B streptococci.

mined MICs were 0.06, 0.03, 0.03, and 0.015 $\mu\text{g/ml}$. One-fourth of the MIC (MIC/4) was the lowest concentration with demonstrable antibiotic activity (Fig. 2). If 99.9% killing is required of the MBC, the MIC was the MBC at 24 h for one of the four bacteria, and $2\times$ MIC was the 24-h MBC for the remaining three bacteria. The rate of killing increased with the drug concentration, but higher concentrations were no longer more bactericidal than $4\times$ MIC (Fig. 2), and this was also true of the 24-h results.

Effect of inoculum. The aforementioned experiments were simultaneously carried out with two 100-fold-different inoculum dilutions of each bacterium, the average concentrations being 6.30 and 4.36 log CFU/ml. On repeated determinations, the MIC for either inoculum remained identical for each of the four organisms. The larger inoculum reached the stationary phase in 4 to 5 h, which explains the difference in the growth curves in the absence of effective antibiotic beyond that time (Fig. 2). The sub-MIC concentrations, MIC/4 and MIC/2, were more inhibitory to the larger inoculum than to the smaller one already during the first hours (Fig. 2) and continued to be so up to 24 h. In the presence of the MIC/4 and MIC/2, the larger inoculum increased on the average slightly over 1 log in 24 h, the individual changes ranging from -0.30 to $+2.70$ log CFU/ml.

In the presence of the MIC of penicillin G, the viable counts of the smaller inoculum did not decrease (Fig. 2), but this concentration was bactericidal to the 100-fold-greater inoculum of each of the four group B streptococci (Fig. 3). The average inoculum-dependent difference was 1.1 log CFU/ml at 2 h, 2.2 at 4 h, and 2.3 at 24 h, corresponding to a 200-fold-greater killing effect by 24 h.

Also, the concentrations exceeding the MIC were generally more bactericidal to the larger than to the smaller inoculum. The differences were small during the first 5 h. At 24 h there was an inverse relationship between the penicillin concentration and its average killing effect against the smaller inoculum: $16\times$ MIC was 0.8 log less effective than $4\times$ MIC, and it was also 1.7 log less effective than $16\times$ MIC against the 100-fold-greater inoculum.

Strain variation. To determine the extent of variation among group B streptococci as to their susceptibility to killing by penicillin G, six isolates were studied. The MICs

were between 0.03 and 0.125 $\mu\text{g/ml}$, and $2\times$ and $4\times$ MICs were used against exponential-phase inocula ranging from 9.5×10^4 to 1.4×10^6 CFU/ml. The differences between the most and the least susceptible organism were 5.6-fold after exposure for 20 min, 14.1-fold after 90 min, 17.4-fold after 6 h, and 100.0-fold after 24 h when $4\times$ MIC was used. The respective ratios were 3.2, 22.4, 22.4, and 199.6 in the presence of $2\times$ MIC of penicillin G. The experiment was partially reproduced four times with the two strains that were most and least susceptible in the presence of a drug concentration (0.5 or 1.0 $\mu\text{g/ml}$) corresponding to $4\times$ MIC or $8\times$ MIC, as judged from simultaneous MIC determinations. The organism that had been associated with slower bactericidal activity was again killed more slowly in all four experiments (Fig. 4). The mean difference in the proportional killing of the two bacteria was 7.8-fold at 6 h in these four experiments.

Effect of growth rate. Based on the growth curves of 32 group B streptococci, the 3 most rapid and the 3 slowest growers were selected for investigation as to whether the rate of killing is related to the rate of multiplication. All tests were carried out in parallel with all six bacteria: the T_2 was determined five times, and the killing curves and MICs were determined three times by using inoculum suspensions derived from exponential-phase cultures. The amount of penicillin to which the bacteria were exposed varied from $6.8\times$ to $10.0\times$ MIC (Table 1), which should cause essentially no variation of the killing curve (Fig. 2). Differences in the rate of killing did not follow differences in T_2 , even if one allows for a correction based on the reverse inoculum effect described in Fig. 2 and 3, i.e., if one assumes a slower than actual rate of killing of isolates K-175 and K-433, which had the greatest inoculum sizes (Table 1).

DISCUSSION

The details of the methods used in broth dilution susceptibility testing of group B streptococci have varied widely, as exemplified by the choice of medium. In the reports cited in this paper, Todd-Hewitt broth has been used by four groups (2, 12, 23, 24), Mueller-Hinton broth by three groups (1, 4, 13, 15–17, 20), Trypticase soy broth (BBL Microbiology Systems) by two groups (9, 18), and Schaedler broth (19) and the Sensititre medium (3) by one group each. Our choice of Todd-Hewitt broth was conservative, and it remains to be seen whether any of our findings were medium dependent.

The effect of penicillin on the viable count of group B streptococci was concentration dependent between the MIC/4 and $4\times$ MIC. Complete killing curves with a range of concentrations, describing the same organism and antibiotic combination, have been rarely reported since the works of Eagle and Musselman (7), whose results on concentration dependence were essentially similar to ours. Wider concentration dependence has been found in the bactericidal activity of penicillin against *Staphylococcus aureus* (5, 8, 22), but the wideness has varied among studies and the strains used, and maximum bactericidal activity has been reached with ca. $10\times$ MIC or less, which is still not dramatically different from the results with group B streptococci.

By definition, the turbidity of the cultures increased in the presence of the MIC/2 and MIC/4 of penicillin. However, in several such cultures the numbers of viable streptococci did not increase or even decreased. An obvious explanation is that simultaneous proliferation and bactericidal activity without lysis took place in the presence of these sub-MIC levels of penicillin.

A third set of concentrations, viz., those exceeding the

FIG. 2. Effect of penicillin G on the numbers of viable group B streptococci in broth at two inoculum concentrations. Each point is the arithmetic mean result for four strains. The penicillin concentrations are expressed in the panels as multiples of the simultaneously determined MICs, and 0 stands for antibiotic-free broth.

one with maximum bactericidal activity, is also relevant in the discussion of concentration dependence. The group B streptococcus is one of the bacterial species showing the so-called Eagle effect or a reduced rate of killing by penicillin at concentrations above the optimal one (5, 7). We did not

study very high concentrations, but data compatible with the Eagle effect were obtained with 8 $\times$ and 16 $\times$ MICs. In conclusion, to characterize the concentration dependence of bactericidal activity, one should include at least the three zones defined here: the sub-MIC concentrations, the 16-fold range with progressively increasing bactericidal activity, and the zone with paradoxically reduced activity at high concentrations.

In the presence of an optimally bactericidal antibiotic concentration, the time dependence of the viable count followed a roughly logarithmic course. However, during the first 2 h the rate of killing was much faster, with a half-life of ca. 20 min, than later on. A reanalysis of previously published data revealed that similar two-phase killing of group B streptococci by penicillin has been recorded by others (1, 6, 12, 16).

Inoculum size may affect susceptibility. At first we will discuss the effect of the inoculum on the MIC. We have previously shown that the MIC of ampicillin against group B streptococci remained the same when the inoculum concentration was varied 10⁴-fold (2), in contrast to the results of Feldman (9), who found over 2,000-fold MICs of both ampicillin and penicillin with a 10³-fold increasing inoculum. This discrepancy became more pronounced when we again found no inoculum effect on the MIC and when McGeary and co-workers reported at least 2,500- to 10,000-fold MICs of penicillin against 10⁴-fold-varying inocula of three group B streptococci (20). The reason for this discrepancy must be studied further.

As to bactericidal activity, an inoculum of 10⁶ CFU/ml was killed more effectively than was one of 10⁴ CFU/ml.

FIG. 3. Effect of the MIC of penicillin G on the numbers of viable group B streptococci in broth at two inoculum concentrations. Each of the four panels stands for one strain.

FIG. 4. Reproducibility of the difference between two group B streptococci (represented by different symbols) as to the rate of killing by penicillin G at a concentration corresponding to either four or eight times the MIC, which was determined simultaneously with each of the four killing experiments, each represented by one panel.

Since low pH reduces killing (12), a pH effect would be expected to have the opposite outcome and therefore does not explain what was found. The reverse inoculum effect was most consistent in the presence of the MIC and at high penicillin concentrations and somewhat less obvious at the optimally bactericidal concentration. An explanation for the apparently new finding is offered by the fact that autolysins are important in the killing of bacteria (21, 26), and heavy inocula obviously provide more autolysin. It is known that extraneous lysis in the medium is sufficient to complete the action of penicillin (11).

Bactericidal activity against either of the two 100-fold-different inocula was equal, if its characterization is reduced to the MBC: 99.9% killing by 24 h was achieved in the presence of $2 \times$ MIC in both cases. Thus, the MBC would have masked the reverse inoculum effect.

Penicillin tolerance by conventional inocula in the order of 10^5 CFU/ml has been regarded as a significant mechanism of

resistance. The present experiments included adequate characterization of 11 group B streptococci, and none was found tolerant as defined by an MCB/MIC ratio of 8 to 32:1 or more. This result was consistent with those reported from several laboratories in the United States, where the prevalence of tolerance has varied from 0 to 8% (13, 16–19), whereas our finding was in striking contrast to the geographically more closely related ones from the Federal Republic of Germany and England, where 5 of 38 (13%), 12 of 21 (57%), and 10 of 14 (71%) tolerant strains were detected (3, 24, 27). The Eagle effect predicts that there should be strains which are not effectively killed by $32 \times$ MIC and more, although the true MBC might be much less. This is an obvious pitfall created by the arbitrary definition of tolerance.

There were reproducible differences among strains in the rate of killing by penicillin. Since the possible correlation between the rates of killing and multiplication was excluded, we could only conclude that the intrinsic variability is determined by some other mechanism, and a likely candidate is the function of autolysins, which we did not study, but which, when deficient, apparently causes antibiotic tolerance or reduced killing (21, 25, 26).

For the in vitro characterization of bactericidal activity in a given antibiotic-organism combination, one must vary at least the antibiotic concentration, the time of exposure, and the inoculum. Even the extensively studied combination of penicillin and group B streptococci cannot be meaningfully reduced to one figure, usually the MBC, and there is a need for further research to fully understand in vitro bactericidal activity. A distant goal should be the identification of in vitro parameters which would be useful in predicting the outcome of antibiotic treatment.

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TABLE 1. Relationship between the rate of multiplication of group B streptococci and the rate of killing by penicillin G

Strain	T_2^a (h)	Inoculum ^b (log CFU/ ml)	Concn of penicillin ^c (multiple of the MIC)	Change in log colony count ^b (log CFU/ml) at:		
				40 min	2 h	6 h
K-433	0.44 ± 0.06	6.4 ± 1.11	6.8	-0.73	-2.42	-3.66
K-4673	0.34 ± 0.03	5.8 ± 0.50	9.7	-0.45	-2.42	-3.43
K-3375	0.33 ± 0.02	6.1 ± 0.80	10.0	-0.81	-2.20	-4.17
K-149	0.33 ± 0.02	5.7 ± 0.73	8.4	-0.66	-1.46	-2.90
K-719	0.32 ± 0.03	5.9 ± 0.42	8.3	-0.77	-2.39	-3.60
K-175	0.31 ± 0.02	6.9 ± 0.47	8.2	-1.28	-2.47	-3.58

^a Mean $\pm$ standard deviation of five determinations, each carried out with the six strains in parallel.

^b Mean $\pm$ standard deviation of three experiments, each carried out with the six strains in parallel.

^c The mean MICs of three determinations done simultaneously with the killing experiments were used to calculate the concentration of penicillin as a multiple of its MIC.

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