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Ampicillin Killing Curve Patterns for Ampicillin-Susceptible Nontypeable *Haemophilus influenzae* Strains by the Agar Dilution Plate Count Method

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Ampicillin killing curve patterns for 20 strains of ampicillin-susceptible nontypeable *Haemophilus influenzae* were determined by the agar dilution plate count method. The paradoxical effect was detected in the 24-h killing curve patterns for each strain. For the biphasic effect, minimum survivor percentages (maximum killing) occurred over a narrow range of ampicillin concentrations immediately above the MIC, with survivor percentages then rising rapidly to peak at ~1-$\log_{10}$-unit increment higher. The 24-h minimum survivor percentages for the 20 strains ranged from ~0.01% (rapid killing) to >10% (slow killing). In comparison with the previous results for typeable strains, the present findings suggest that nontypeable stains are, on average, killed much more slowly. Based on the initial 24-h killing curve patterns for the 20 strains, 4 strains were selected as putative representatives of the range of bactericidal responses encountered. These strains were then studied to examine the reproducibility of the 24-h patterns and to determine sequential killing curves. These patterns were found to be reproducible and served to characterize the relative killing responses of the strains. In the sequential studies of three of the four strains, tiny colonies having the gross and microscopic characteristics of L-forms were found to be present on the agar dilution plate count plates prior to the application of penicillinase at 48 and 72 h. Such colonies reverted to vegetative forms within 24 to 48 h after application of penicillinase to the panels. Of particular interest was the observation that the paradoxical effect was manifested both by the L-form colonies and by the reverted vegetative colonies. The late development of L-forms was observed for both rapidly and slowly killed strains.

In a previous study (35) we described ampicillin killing curve patterns for type b *Haemophilus influenzae* strains as determined by the agar dilution plate count (ADPC) method. Killing curve patterns were found to be strain dependent and ranged from strains that were killed slowly to others that were killed rapidly. The paradoxical effect (7) was demonstrated at some time during the killing sequence for each strain studied. We present here a similar study of ampicillin killing curve patterns for ampicillin-susceptible nontypeable *H. influenzae* strains.

MATERIALS AND METHODS

Study design. Twenty ampicillin-susceptible nontypeable *H. influenzae* strains were studied. On retrieval from stock culture, each strain was confirmed to by nontypeable *H. influenzae* and ampicillin susceptible by standard microbiological methods. Several 24-h killing curve patterns were then determined by the ADPC method for each strain (35). On the basis of the 24-h patterns, several putative rapidly to slowly killed strains were studied further to determine the reproducibility of the 24-h patterns and to determine patterns of sequential killing after various times of ampicillin action.

***H. influenzae* strains.** Of the 20 ampicillin-susceptible nontypeable *H. influenzae* isolates studied, 15 were obtained through the courtesy of Timothy F. Murphy (17) of the Department of Medicine, State University of New York, Buffalo, and 5 were obtained from the stock culture collection of the St. Paul-Ramsey Medical Center Clinical Microbiology Laboratory. Stock cultures were prepared in 5% defibrinated sheep blood and maintained at −70°C. Strains were retrieved from stock culture by two 24-h passages on chocolate Mueller-Hinton (prepared from Mueller-Hinton agar [MHA; BBL Microbiology Systems, Cockeysville, Md.]) that was incubated at 35°C in a humidified 5% CO_2 atmosphere.

AD susceptibility tests. Ampicillin MICs were determined by an agar dilution (AD) replicator method in accordance with the recommendations of the National Committee for Clinical Laboratory Standards (18) by using both chocolate MHA and the recently described (12) *H. influenzae* growth and susceptibility test medium (HTM) agar. Isolates were judged to be susceptible to ampicillin if MICs on both chocolate and HTM agar were ≤2 μg/ml.

Ampicillin stability in HTM agar. In previous studies (34, 35) we encountered some loss of ampicillin activity in ADPC plates made with MHA and 1% supplement C (Difco Laboratories, Detroit, Mich.) as a consequence of various lots of supplement C containing small amounts of β-lactamase. To avoid this problem in the present study, ADPC panels were prepared with the recently introduced HTM (12). The stability of ampicillin in HTM agar under storage (4°C) and incubation (humidified 5% CO_2 atmosphere at 35°C) conditions was studied as follows. AD panels were prepared with HTM containing ampicillin in twofold dilution concentrations ranging from 0.06 through 64 μg/ml. The stability of ampicillin under storage conditions at 4°C was examined by removing panels after 0, 1, 2, 6, 7, 14, 21, and 42 days of

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storage, at which time they were brought to room temperature, inoculated by the AD replicator method with *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922, and then incubated at 35°C in a humidified 5% CO_2 atmosphere for 16 to 20 h. The MICs were then determined. The stabilities of ampicillin panels under incubation conditions were examined by inoculations with *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 on the day of preparation and after 24, 48, and 72 h of incubation at 35°C in a 5% CO_2 humidified atmosphere. MICs were then determined 16 to 20 h after panel inoculation.

Preparation and standardization of ADPC inoculum. Material from a number of colonies on the second stock retrieval plate was inoculated into 5 ml of HTM broth (12) contained in a borosilicate glass tube (16 by 125 mm), which was then incubated at 35°C in air until the broth approached the density of a 0.5 McFarland standard. This procedure was previously shown (35, 39) to produce a log-phase culture of $\sim 3 \times 10^8$ CFU/ml. The culture was then diluted 1:10, 1:100, and so on with Mueller-Hinton broth to produce the appropriate inoculum densities as needed for the ADPC inoculation. The actual density of each inoculum preparation was determined by pipetting 0.1 ml onto the surface of each of three HTM agar plates, which was then thoroughly streaked with a bacteriological loop in three opposing directions to disperse the inoculum evenly over the entire surface of each plate. Plates were allowed to dry for ~15 min, two of the plates were overlaid with 10 ml of molten (48°C) HTM agar, and the preparations were then incubated at 35°C in an humidified 5% CO_2 atmosphere for 48 to 72 h, at which time the colonies were counted. The colony counts were then compared for the overlaid and the nonoverlaid plates and then averaged for calculation of the actual inoculum density.

Preparation and interpretation of ADPC panels. ADPC panels were prepared (35) with HTM agar containing ampicillin in twofold dilution concentrations ranging from 0.25 through 128 μg/ml and were used within 1 week of preparation, most often on the day they were made. Immediately after preparing the standardized inoculum, 0.050 ml was pipetted onto the surface of each plate and streaked with a bacteriological loop to disperse thoroughly the inoculum evenly over the entire surface. Inoculated plates were allowed to dry for ~15 min, at which time each plate was overlaid with 10 ml of molten (48°C) HTM agar containing an analogous concentration of ampicillin. Panels were prepared in duplicate for each isolate and were then incubated at 35°C in a 5% CO_2 humidified atmosphere for 24 h, at which time MICs were determined. Analogous to other dilution methods, the MIC on ADPC panels was defined as the minimum ampicillin concentration in the ADPC panel for and above which bacterial growth was absent when the panel was examined after 24 h and before the application of β-lactamase. In actual practice, colony counts on the MIC plate were virtually absent at the 24-h reading, with MICs occasionally increasing one dilution step on prolonged incubation. After determination of the MIC, each plate was overlaid with 1.0 ml of a 1:10 dilution of Penase (Difco) in sterile deionized water to inactivate the ampicillin. Plates were then reincubated for 48 h, at which time colony counts were made to determine the number of CFU that were able to survive the ampicillin action. For some slow-growing strains, plates were incubated for an additional 24 h to allow for the development of colonies large enough for accurate counting. The average colony count from the duplicate plates was used to calculate the percentage of surviving CFU at each ampicillin concentration. The paradoxical effect (7) was defined as a progressive increase in the percentages of persisting colonies for two or more twofold dilution steps above the MIC. Paradoxical trough and peak survivor percentages were defined as the lowest and highest numbers of colonies encountered, respectively, in the biphasic paradoxical pattern. The trough survivor percentage thus represented the maximum killing effect observed for ampicillin concentrations immediately above the MIC in the so-called zone of optimum bactericidal action described by Eagle and Musselman (7). Conversely, the peak survivor percentage represented the minimum killing effect observed for ampicillin concentrations above the optimal killing zone. By using the methods and definitions described above, 24-h killing curve patterns were determined for each of the 20 nontypeable *H. influenzae* strains.

To assess the reproducibility of the 24-h patterns, several putative slowly to rapidly killed strains were selected on the basis of their different trough survivor percentages for reproducibility studies. For each strain, reproducibility was investigated by determining six to eight 24-h killing curve patterns by using inoculum preparations made from separate retrievals from stock cultures. Strain dependency and the ability to detect the paradoxical effect were also examined by determining sequential killing curve patterns representing various times of ampicillin action. To do this, a sufficient number of ADPC panels were prepared for each isolate so that duplicate panels could be overlaid with Penase to inactivate ampicillin at 6, 12, 24, 48, and 72 h. Inocula were sometimes adjusted to 1- or 2-$\log_{10}$-unit increments above or below the standard $\simeq 1.5 \times 10^5$ CFU per plate in order to provide statistically optimal counts for some slowly and rapidly killed strains, for panel concentration zones showing large differences in trough and peak survivor percentages, and for sequential curves representing short and long times of ampicillin action.

For all of the ADPC procedures, internal quality control was provided by comparing the MIC on ADPC panels with that determined for the isolate by the AD chocolate MHA and HTM agar methods. For all trials, the MIC on ADPC panels was found to be equal to or slightly less than the values obtained by the AD methods.

RESULTS

Ampicillin stability in HTM agar. Ampicillin MICs determined in AD panels stored at 4°C for 0, 1, 2, 6, 7, 14, 21, and 42 days were 0.5, 0.5, 0.5, 1, 0.5, 0.5, 0.5, and 1 μg/ml, respectively, for *S. aureus* ATCC 29213 and 8, 4, 8, 8, 8, 8, 8, and 8 μg/ml, respectively, for *E. coli* ATCC 25922, thus indicating a rather remarkable stability of ampicillin under storage conditions. MICs determined by inoculating panels after incubation times of 0, 24, 48, and 72 h at 35°C in humidified 5% CO_2 atmosphere were 0.5, 0.5, 0.5, and 1 μg/ml, respectively, for *S. aureus* ATCC 29213 and 4, 8, 8, and 8 μg/ml, respectively, for *E. coli* ATCC 25922, indicating an acceptable stability of ampicillin under prolonged conditions of incubation in ADPC sequential killing curve studies extended over several days.

MICs for nontypeable *H. influenzae* strains. Table 1 presents a summary of the ampicillin MICs determined for the 20 nontypeable *H. influenzae* strains by the AD method with panels prepared with chocolate MHA and HTM agar and by the ADPC method with panels prepared with HTM agar. ADPC MICs were predominantly equivalent to or 1 dilution step lower than AD MICs, with 9, 7, 2, 1, and 1 being 0, −1,

TABLE 1. Summary of ampicillin MICs for 20 ampicillin-susceptible nontypeable *H. influenzae* strains[a]

Method	No. of strains for which ampicillin MICs (μg/ml) were:						
	0.03	0.06	0.12	0.25	0.5	1	2
AD-MHA-Choc				3	12	3	2
AD-HTM				3	12	3	2
ADPC-HTM	1	1	2	6	7	2	1

[a] MICs were determined by the AD method with both chocolate MHA (AD-MHA-Choc) and HTM (AD-HTM) agars and by the ADPC method with HTM agar (ADPC-HTM).

−2, −3, and −4 twofold dilution steps lower, respectively, than the corresponding AD MICs. MICs for several strains showed a marked inoculum dependency that required a carefully prepared inoculum of 10^4 CFU per spot or less, as recommended (4, 30) for haze-forming strains of *H. influenzae*. For such strains, Gram-stained preparations of the apparent surface growth on AD plates having concentrations above the MIC showed that essentially all of the cells had morphologic features of cell wall-defective forms. These observations were similar to those described previously (4, 24, 30) and are discussed below in relationship to the killing curve patterns for the 20 strains examined in this study.

Killing curve patterns at 24 h. The paradoxical effect was observed in the 24-h killing curve patterns for all 20 strains. Maximum killing (trough survivor percentages) for each of the 20 strains occurred over a narrow range of ampicillin concentrations immediately above the MIC. For concentrations above the trough, the majority of strains showed survivor percentages that increased rapidly, with peak percentages often being 1 or more $\log_{10}$ increments higher. For all 20 strains, the 24-h patterns formed an evenly distributed array ranging from that for the most slowly killed strain (trough survivor percentage, >10%) to that for the most rapidly killed strain (trough survivor percentage, ≈0.01%).

The reproducibility of the 24-h patterns is illustrated in Fig. 1A through 4A, which represent clusters of six to eight independently determined patterns for strains 1034, 3198, 3524, and 2019, respectively, and which represent the range of strain-dependent bactericidal responses that were observed for the 20 strains. For strain 1034 (Fig. 1A), the pattern cluster was tight, with trough percentages exceeding 10% and peak percentages approaching 100%, thus indicating a remarkable ability of this strain to survive under the action of ampicillin. For strain 3198 (Fig. 2A), the pattern cluster was again tight, with trough percentages exceeding 1% and peak percentages being above 10%, thus indicating that the strain has a slow bactericidal response to ampicillin but one that is somewhat greater than that of strain 1034. For

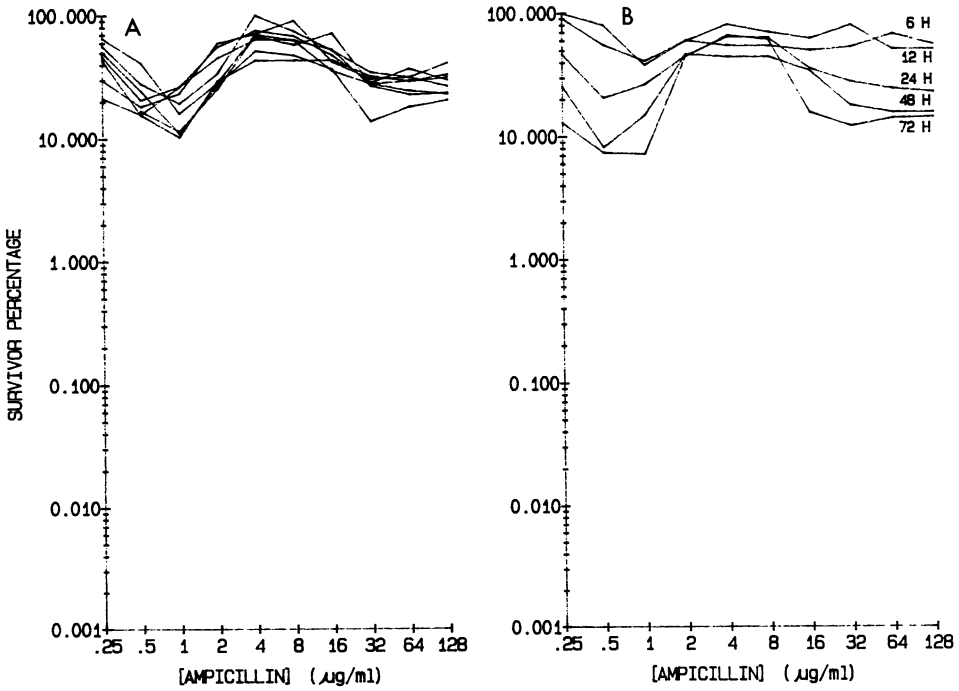

FIG. 1. (A) Ampicillin killing curve patterns for strain 1034 after ampicillin action for 24 h determined in eight separate trials. (B) Sequential ampicillin killing curve patterns for strain 1034. Curves designated 6, 12, 24, 48, and 72 h represent the percentages of inocula surviving as viable CFU after the indicated duration of ampicillin action. Data for the sequential patterns represent a single trial from stock culture for which the survivor percentages were calculated from duplicate panels prepared for each time point. The ordinates represent the percentage of the inoculum that survive as viable CFU after ampicillin action. The abscissas represent ampicillin concentrations in the ADPC plates.

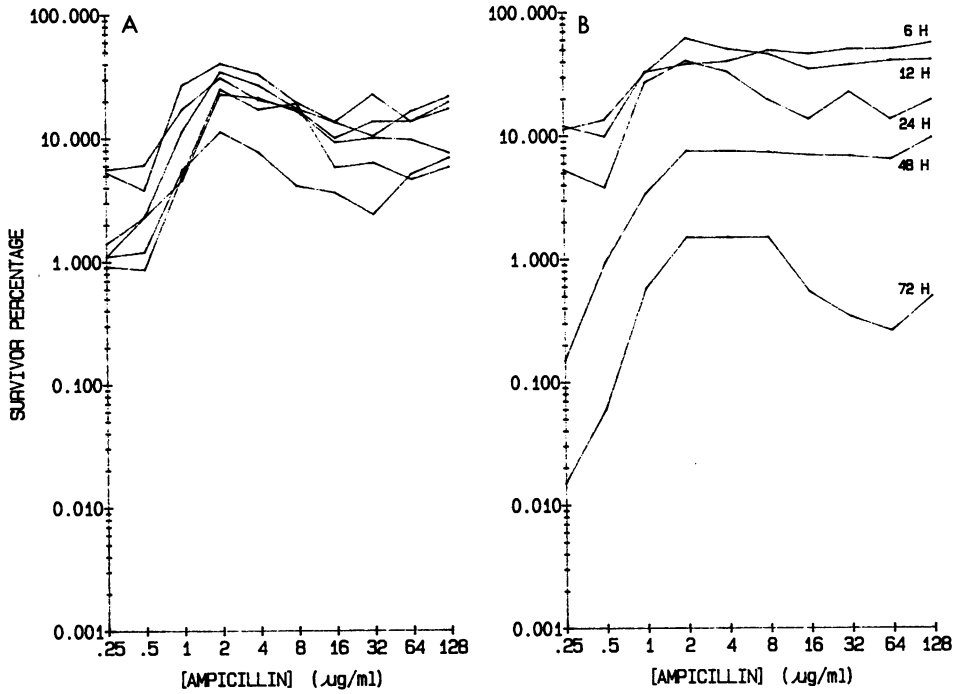

FIG. 2. (A) Ampicillin killing curve patterns for strain 3198 after ampicillin action for 24 h determined in six separate trials. (B) Sequential ampicillin killing curve patterns for isolate 3198. Curves designated 6, 12, 24, 48, and 72 h represent the percentages of inocula surviving as viable CFU after the indicated duration of ampicillin action. Data for the sequential patterns represent a single trial from stock culture for which the survivor percentages were calculated from duplicate panels prepared for each time point. The ordinates and abscissas are as described in the legend to Fig. 1.

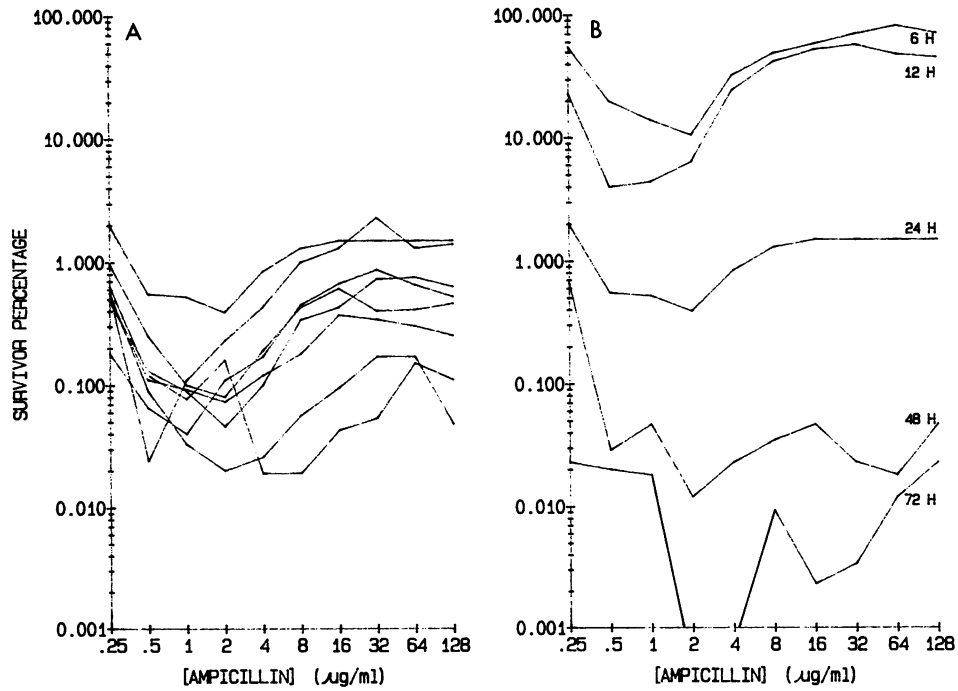

FIG. 3. (A) Ampicillin killing curve patterns for strain 3524 after ampicillin action for 24 h determined in eight separate trials. (B) Sequential ampicillin killing curve patterns for isolate 3524. Curves designated 6, 12, 24, 48, and 72 h represent the percentages of inocula surviving as viable CFU after the indicated duration of ampicillin action. Data for the sequential patterns represent a single trial from stock culture for which the survivor percentages were calculated form duplicate panels prepared for each time point. The ordinates and abscissas are as described in the legend to Fig. 1.

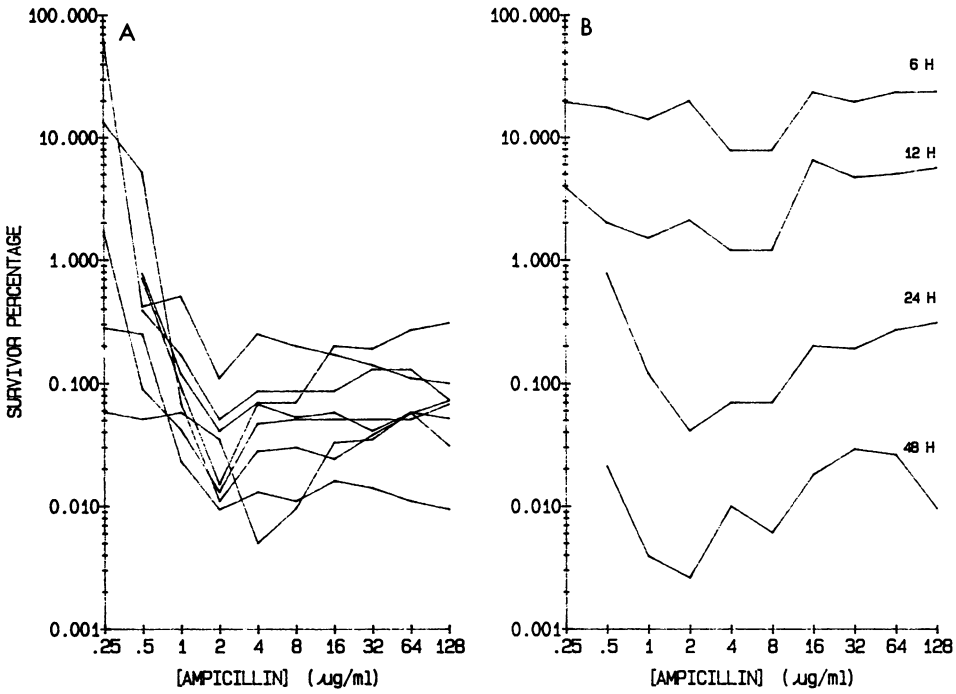

FIG. 4. (A) Ampicillin killing curve patterns for strain 2019 after ampicillin action for 24 h determined in eight separate trials. (B) Sequential ampicillin killing curve patterns for isolate 2019. Curves designated 6, 12, 24, 48, and 72 h represent the percentages of inocula surviving as viable CFU after the indicated duration of ampicillin action. Data for the sequential patterns represent a single trial from stock culture for which the survivor percentages were calculated from duplicate panels prepared for each time point. The ordinates and abscissas are as described in the legend to Fig. 1.

strain 3524 (Fig. 3A), the pattern cluster showed curves divergent over a 1-$\log_{10}$-unit increment, with trough and peak survivor percentages averaging about 0.1 and 1%, respectively. For isolate 2019 (Fig. 4A), the pattern cluster was slightly more divergent than that for strain 3524, with trough percentages being between 0.01 and 0.1% and peak percentages being approximately 0.5-$\log_{10}$-unit increment higher. Although the pattern clusters for strains 3524 and 2019 (representing the more rapidly killed strains) were more divergent than those encountered for strains 1034 and 3198 (representing the more slowly killed strains), they were deemed sufficient to characterize the relative response of these strains to the bactericidal action of ampicillin.

Sequential killing curves. The sequential killing curve patterns determined after various times of ampicillin action for strains 1034, 3198, 3524, and 2019 are presented in Fig. 1B through 4B, respectively. For strain 1034 (Fig. 1B), trough survivor percentages after 6, 12, 24, 48, and 72 h of ampicillin action were ~38, 41, 21, 8, and 7%, respectively. With extended ampicillin exposure, the paradoxical effect became more prominent, and trough percentages dropped; however, peak percentages remained high, with 50 to 80% of the original inoculum surviving. On examination of panels at 48 h, immediately before Penase application, tiny colonies were perceptible in the plates containing ampicillin at greater than or equal to the MIC. For the 72-h preparations, before the addition of Penase, colonies were more apparent and the paradoxical effect was perceivable for plates with the higher concentrations of ampicillin. Close scrutiny of colony morphology showed that the features of some were characteristic of L-form colonies, and random material selected for Gram-stained colonies showed no normal coccobacillary forms but only cells with the features of L-forms. In contrast, when colonies were examined 24 h after the addition of Penase at 48 and 72 h, Gram-stained preparations showed reversion of the L-form colonies to vegetative colonies, as indicated by the presence of numerous small gram-negative bacilli that developed and spread from the edge of granular colonies along with normal coccobacillary forms that were distributed elsewhere. These findings suggest that most if not all of the surviving CFU originated initially as osmotically stable cell wall-defective forms and not from cells with a normal coccobacillary morphology. Because growth of the small colonies was only discernible on panels incubated for 48 h or more, the possibility that the phenomenon might be caused by inactivation of ampicillin, either because of alterations in the medium produced by addition of the inoculum or by the production of β-lactamase by the surviving fraction, was investigated. Inoculated panels showing discernible colony growth after 48 and 72 h of incubation were spot inoculated with $\simeq 10^4$ CFU of *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 and reincubated at 37°C for 24 h to assess potential ampicillin degradation as indicated by an increase in MICs. MICs were found to be the same as those determined earlier for ampicillin under conditions of incubation and were identical to those known for the *S. aureus* and *E. coli* strains. Materials selected from colonies on plates after 48 and 72 h of incubation without β-lactamase showed no evidence of β-lactamase production, as tested by the chromogenic cephalosporin method. Figure 2B shows the sequential curves for isolate 3198. Trough survivor percentages for ampicillin action at 6 and 12 h each approximated 10%, with peaks exceeding 50% survival. Trough percentages for ampicillin action times of 24, 48, and 72 h were ~4,

0.2, and 0.2%, respectively, indicating that the strain was somewhat more rapidly and progressively killed than strain 1034 was. The paradoxical effect was distinct for all the curves, with peaks increasing proportionately over the time of ampicillin action. Examination of 48- and 72-h preparations prior to the addition of Penase showed no distinct tiny colonies. This situation suggests that although L-forms may have been present, growth in the presence of ampicillin was insufficient to form colonies that were large enough for detection, even though the strain was killed slowly. Figure 3B presents the sequential killing curves for strain 3524. Compared with the strains described above, the patterns indicate more rapid and progressive killing of strain 3524 for the specific times of ampicillin action, and the paradoxical phenomenon was again evident in each of the patterns. As with strain 1034, and unlike strain 3198, tiny colonies were observed on the 48- and 72-h preparations before the addition of Penase. These tiny colonies showed the morphologic features of cell wall-defective colonies, and Gram-stained material showed no normal coccobacillary forms but only large irregular and spherical forms intermixed with smaller granular bodies that were significantly larger than normal bacilli and that were deemed to be characteristic of cell wall-defective microorganisms.

Figure 4B presents the sequential patterns for strain 2019. Compared with the other three isolates described above, each sequential pattern showed distinctly lower trough survivor percentages, with the paradoxical effect being present for each curve. As with strains 1034 and 3524, tiny cell wall-defective colonies were also observed in the 48- and 72-h preparations, before the addition of Penase.

DISCUSSION

We previously developed the ADPC method (34, 36, 38) to provide an alternative to broth dilution methodology for assessing bactericidal action. The uniqueness of the ADPC method is that the inoculum CFU is immobilized in an agar gel matrix during the action of and after the inactivation of the antimicrobial agent, thus potentially avoiding variations in minor technical factors that are known to influence markedly the results of broth dilution methods. Using the ADPC method, we studied the bactericidal action of oxacillin against strains of *S. aureus* and ampicillin against strains of *H. influenzae* type b. For each of these genera, we found the bactericidal action to be strain dependent and ranged from strains that were killed rapidly to others that were killed more slowly. Of special interest was the finding that the paradoxical effect was manifested at some time during the killing sequence of each strain.

The present investigation was undertaken to determine whether nontypeable strains might respond differently from typeable strains to the bactericidal action of ampicillin and to provide a base of reference for a study in progress on the bactericidal action of ampicillin on a unique group of nontypeable strains that are ampicillin resistant but that do not produce β-lactamase. The results of the present study indicate that ampicillin-susceptible nontypeable strains are also killed in a strain-dependent fashion and that the paradoxical effect can be found for each strain. In anticipation of comparison of the results of the present study with those of the previous study (35) of typeable *H. influenzae* strains and with the knowledge that results can be media dependent (13, 20, 21), killing curves prepared for typeable and nontypeable *H. influenzae* strains by using ADPC panels prepared with both HTM agar and MHA–1% supplement C showed (unpublished data) essentially equivalent or slightly lower patterns with HTM agar, thus providing assurance that the results of the two studies might be validly compared. From the results of the two studies, nontypeable strains appear to be killed more slowly. In the present investigation, the reproducibility studies of the 24-h killing curve patterns showed tight pattern clusters for the more slowly killed strains. For the more rapidly killed strains, 24-h patterns were more divergent but were sufficient as a cluster to characterize the bactericidal response of a strain. The range of strain-dependent bactericidal responses observed for the 20 strains (trough survivor percentages, $\approx$0.01 to >10%), the universal presence of the paradoxical effect, and the divergence of 24-h patterns for the more rapidly killed strains serve to point out the danger of using only one 24-h pattern to assess bactericidal action and the ineffectiveness of using arbitrary break points (such as 0.1% survival) and artificial indices (such as the MBC or the MBC/MIC) to assess bactericidal response. A question exists as to whether bactericidal response can best be judged by 24-h patterns or by patterns determined earlier or later in the killing sequence. The 6-, 12-, 48-, and 72-h sequential patterns in the present study essentially paralleled the results of those determined at 24 h and seemed to show no advantage one way or the other. It is possible, however, particularly for the more rapidly killed strains, that the reproducibility of patterns might be improved at the 6- or 12-h time points.

The mechanisms by which microorganisms survive the action of antimicrobial agents are poorly understood and remain debatable. Although the production of osmotically stable L-forms has been generally discounted (9–11, 26) as a significant contribution to the in vitro survival of microorganisms, as determined by killing curves, findings of this study and those of other studies discussed below suggest that the subject should be carefully reexamined. Our observations indicated that the long-term survival of strains 1034, 3524, and 2019 were due, at least in part, to the production of osmotically stable L-forms that remained viable and even multiplied under the influence of ampicillin and that regrew as vegetative forms after ampicillin was inactivated. Apropos are the previous studies by Roberts et al. (24), Bottone et al. (4), and Sykes et al. (30) relating to the problems encountered in defining susceptibility test inhibition break points. Certain strains of *H. influenzae* were observed to have a proclivity to form a hazy growth in broth dilution tubes, on agar dilution plates, and within inhibition zones on disk agar diffusion tests. For concentrations immediately above the MIC, microscopic examination of material from hazy growths generally showed the presence of enlarged filamentous, nonseptate or often multiseptate forms with lateral, irregularly sized outpouchings along the filaments. In contrast, for progressively higher concentrations than those immediately above the MIC, microscopic examination showed variously sized and irregularly shaped, large spherical bodies in granular forms without the presence of filaments or normal coccobacillary forms. Subculture of the hazy material to PPLO agar showed the development of tiny L-form colonies that revealed by microscopy large and irregular spherical bodies compatible with spheroplasts. When such colonies were subcultured onto chocolate agar, typical vegetative colonies developed that revealed by microscopy the normal gram-negative coccobacillary forms of *H. influenzae*. The haze phenomenon was shown to be inoculum dependent, and inhibition break points were mark-

edly improved by using inoculum densities of 10^4 CFU/ml or lower or by microscopically examining growth to determine the break point at which normal coccobacillary forms could not be found. Results of scanning electron microscopic studies presented by Klein and Luginbuhl (15) provided confirmatory evidence that growth at concentrations greater than the MIC was due to the presence of cell wall-defective forms. Additional information in support of this concept was provided by Yourassowsky and associates (40), who studied the action of cefamandole on a β-lactamase-producing strain of *H. influenzae*. Concomitant densitometric and morphologic studies of an actively growing culture acted upon by cefamandole at a supra-MIC (10 μg/ml) showed the rapid development of spherical cell wall-defective forms that, as cefamandole was inactivated by β-lactamase production, increased in number and eventually reverted to normal bacillary forms. Those investigators noted the potential of osmotically stable L-forms to complicate bactericidal studies, the extent of which may vary with strain, medium, and inoculum size. Evidence that the production of osmotically stable L-forms might be a significant in vivo mechanism permitting *H. influenzae* to survive the action of β-lactam agents has recently been presented by Roberts and associates (23). Using a special medium supplemented with *N*-acetylglucosamine to study bronchial secretions, they readily isolated L-forms that promptly reverted to typical *H. influenzae* vegetative forms on subculturing. They proposed that under the influence of lysozyme, β-lactam agents, or both in tracheal bronchial secretions, spheroplast-forming strains of *H. influenzae* might be responsible for the recurrence and chronicity of tracheobronchitis.

A number of researchers other than those mentioned above have investigated the inhibitory and bactericidal actions of ampicillin and other agents on *H. influenzae* strains, but they did not include concomitant morphologic observations and yielded a paucity of information on killing kinetics. The effect of inoculum size on ampicillin and some other β-lactam MICs for *H. influenzae*, particularly for β-lactamase-producing strains, has been reported and discussed previously (5, 16, 28, 31). Several studies on the bactericidal action of ampicillin (6, 25, 33) have found in general that strains undergo 99.9% killing in 24 h by supra-MICs. Bergeron and colleagues (2) found that ampicillin-susceptible strains undergo 99.9% killing in 24 h by 2 μg of ampicillin, moxalactam, or chloramphenicol per ml, with cefamandole showing less bactericidal action against 11 strains. Simard and Bergeron (28) found 4 of 87 ampicillin-susceptible *H. influenzae* strains to be killed slowly (MBC and MIC, ≥32 μg/ml) by cefoperazone, moxalactam, cefotaxime, and cephalothin. Those investigators mentioned the possibility that slow killing might be due to the production of cell wall-defective forms. Bergeron and LaVoie (3), using the MBC as a measure of killing, found 9 of 165 ampicillin-susceptible *H. influenzae* strains to be killed slowly (MBC and MIC, ≥32 μg/ml) with stationary-phase inocula, with only four strains being killed slowly with exponential-phase inocula. Broth dilution plate count killing curves determined at 2, 6, 12, and 24 h with a single ampicillin concentration of 2 μg/ml were said to correlate well with MBCs. The reproducibility of the killing curves was not mentioned, and the paradoxical effect, unfortunately, could not be observed because of the use of a single ampicillin concentration.

Although the paradoxical effect was observed over 40 years ago (7, 8, 14), the biochemical mechanisms responsible for the phenomenon remain unknown and speculative. The effect has been observed primarily for cell wall-active agents against a variety of microorganisms, with the effect being observed inconsistently by the broth dilution methodology (1, 27). As far as we can tell, the paradoxical effect was not observed for *H. influenzae* prior to the elegant study by Yourassowsky and associates (41). By using a unique triple-agar-layer disk diffusion method in which the area of the antimicrobial gradient zone around the disk was monitored by optical-galvanometric densitometry for quantitative growth after inactivation of ampicillin by β-lactamase, a variety of gram-positive and gram-negative bacteria were studied for the presence of the paradoxical effect. By this agar-based method, a high incidence of the paradoxical phenomenon was noted for a number of species, with the effect being found for 10 of 10 *H. influenzae* strains. These findings, in conjunction with those of our previous (35) and present studies, may indicate that the phenomenon is enhanced or more easily detected by agar-based systems. In a study of the bactericidal action of moxalactam against *S. aureus*, Nelson and Washington (19) reported a close association of the paradoxical effect with strains that appear to be killed slowly. Taylor and associates (32), in a study of the bactericidal action of oxacillin against *S. aureus*, also observed that the paradoxical effect was manifested most frequently for the more slowly killed strains. More recently (22, 29), the association of slow killing and the paradoxical effect has been described for penicillin against a variety of streptococci, with the paradoxical effect being said to be a potential marker for so-called tolerant strains. In reviewing the methodology and data of these studies, we have suggested (37) that the observed association of the paradoxical effect and slow killing was due to the fact that plate counts for rapidly killed strains were most likely too low and too imprecise to detect the paradoxical phenomenon, whereas for slowly killed strains, plate counts were sufficiently high and statistically appropriate to enable the effect to be consistently detected.

In considering the results of our own studies and those of others in measuring bactericidal action, more questions seem to be raised than answered. The principal morphologic form(s) (cell wall-defective forms, normal vegetative forms in suspended animation, or other forms) in which bacteria exist while surviving the in vitro action of an antimicrobial agent has yet to be established. To assess the bactericidal response, strains of a number of genera need to be studied by sensitive methods in order to confirm or refute whether the paradoxical effect is a universal phenomenon. Studies to elucidate the basic mechanisms responsible for the paradoxical effect are needed and should address the putative roles of the differential inhibition of cell wall-active enzymes by various antimicrobial agent concentrations and the efficiencies at which various concentrations of an agent promote the development of osmotically stable L-forms. There is a distinct need for standardization of test methodologies to enable the accurate and reproducible characterization of a bactericidal response, and any such characterization should include not only the time of action of the antimicrobial agent and the percentages of surviving bacteria for a full spectrum of concentrations of antimicrobial agents but also specific details of the method, media, temperature, pH, and phase of growth of the inoculum preparation. It will be important to establish reference strains that represent rapid, intermediate, and slow bactericidal responses for the strains of various genera in order to resolve the existing controversies related to the clinical meaningfulness of strains that are killed rapidly or slowly. In the quest for answers to some of these questions, a healthy skepticism should be maintained in

considering the results of past and future studies of bactericidal action.

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LITERATURE CITED

1. **Annear, D. I.** 1982. Paradoxical effect of antibiotics. II. Comment and bibliography. J. Antimicrob. Chemother. **10**:260–262.
2. **Bergeron, M. G., S. Claveau, and P. Simard.** 1981. Limited in vitro activity of cefamandole against 100 beta-lactamase and non-beta-lactamase-producing *Haemophilus influenzae* strains: comparison of moxalactam, chloramphenicol, and ampicillin. Antimicrob. Agents Chemother. **19**:101–105.
3. **Bergeron, M. G., and G. Y. Lavoie.** 1985. Tolerance of *Haemophilus influenzae* to β-lactam antibiotics. Antimicrob. Agents Chemother. **28**:320–325.
4. **Bottone, E. J., Z. Brandman, and S. S. Schneierson.** 1976. Spheroplasts of *Haemophilus influenzae* induced by cell wall-active antibiotics and their effect upon the interpretation of susceptibility tests. Antimicrob. Agents Chemother. **9**:327–333.
5. **Bulger, R. R., and J. A. Washington II.** 1980. Effect of inoculum size and β-lactamase production on in vitro activity of new cephalosporins against *Haemophilus* species. Antimicrob. Agents Chemother. **17**:393–396.
6. **Cole, F. S., R. S. Daum, L. Teller, D. A. Goldmann, and A. L. Smith.** 1979. Effect of ampicillin and chloramphenicol alone and in combination on ampicillin-susceptible and -resistant *Haemophilus influenzae* type B. Antimicrob. Agents Chemother. **15**:415–419.
7. **Eagle, H., and A. D. Musselman.** 1948. The rate of bactericidal action of penicillin in vitro as a function of its concentration, and its paradoxically reduced activity at high concentrations against certain organisms. J. Exp. Med. **88**:99–131.
8. **Garrod, L. P.** 1945. The action of penicillin on bacteria. Br. Med. J. **1**:107–110.
9. **Greenwood, D., and F. O'Grady.** 1970. Trimodal responses of *Escherichia coli* and *Proteus mirabilis* to penicillin. Nature (London) **228**:457–458.
10. **Gunnison, J. G., M. A. Fraher, and E. Jawetz.** 1964. Persistence of *Staphylococcus aureus* in penicillin *in vitro*. J. Gen. Microbiol. **35**:335–349.
11. **Hamburger, H., and J. Carleton.** 1968. Staphylococcal spheroplasts as persisters, p. 221–229. *In* L. B. Guze (ed.), Microbiol protoplasts, spheroplasts and L-forms. The Williams & Wilkins Co., Baltimore.
12. **Jorgensen, J. H., J. S. Redding, L. A. Maher, and A. W. Howell.** 1987. Improved medium for antimicrobial susceptibility testing of *Haemophilus influenzae*. J. Clin. Microbiol. **25**:2105–2113.
13. **Kim, K. S., R. N. Yoshimori, D. T. Imagawa, and B. F. Anthony.** 1979. Importance of medium in demonstrating penicillin tolerance by group B streptococci. Antimicrob. Agents Chemother. **16**:214–216.
14. **Kirby, W. M. M.** 1945. Bacteriostatic and lytic actions of penicillin on sensitive and resistant staphylococci. J. Clin. Invest. **24**:165–169.
15. **Klein, R. D., and G. H. Luginbuhl.** 1977. Ampicillin-induced morphological alterations of *Haemophilus influenzae* type b. Antimicrob. Agents Chemother. **11**:559–562.
16. **Marks, M. I., and G. Weinmaster.** 1975. Influences of media and inocula on the in vitro susceptibility of *Haemophilus influenzae* to co-trimoxazole, ampicillin, penicillin, and chloramphenicol. Antimicrob. Agents Chemother. **8**:657–663.
17. **Murphy, T. F., and M. A. Apicella.** 1987. Nontypable *Haemophilus influenzae*: a review of clinical aspects, surface antigens, and the human immune response to infection. Rev. Infect. Dis. **9**:1–15.
18. **National Committee for Clinical Laboratory Standards.** 1985. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M-7A. National Committee for Clinical Laboratory Standards, Villanova, Pa.
19. **Nelson, R. E., and J. A. Washington II.** 1981. Paradoxic and tolerant effects of moxalactam on *Staphylococcus aureus*. J. Infect. Dis. **144**:178.
20. **Norden, C. W., and E. Keleti.** 1981. Antibiotic tolerance in strains of *Staphylococcus aureus*. J. Antimicrob. Chemother. **7**:599–605.
21. **Peterson, L. R., D. N. Gerding, W. H. Hall, and E. A. Schierl.** 1978. Medium-dependent variation in bactericidal activity of antibiotics against susceptible *Staphylococcus aureus*. Antimicrob. Agents Chemother. **13**:665–668.
22. **Powley, L., J. Meeson, and D. Greenwood.** 1989. Tolerance to penicillin in streptococci of viridans group. J. Clin. Pathol. **42**:77–80.
23. **Roberts, D., E. Higgs, A. Rutman, and P. Cole.** 1984. Isolation of spheroplastic forms of Haemophilus influenzae from sputum in conventionally treated chronic bronchial sepsis using selective medium supplemented with N-acetyl-D-glucosamine: possible reservoir for re-emergence of infection. Br. Med. J. **289**:1409–1412.
24. **Roberts, D. E., A. Ingold, S. V. Want, and J. R. May.** 1974. Osmotically stable L forms of *Haemophilus influenzae* and their significance in testing sensitivity to penicillins. J. Clin. Pathol. **27**:560–564.
25. **Rocco, V., and G. Overturf.** 1982. Chloramphenicol inhibition of the bactericidal effect of ampicillin against *Haemophilus influenzae*. Antimicrob. Agents Chemother. **21**:349–351.
26. **Seeberg, S., and J. E. Brorson.** 1975. Prolonged survival of non-multiplying *Staphylococcus aureus* cells undergoing autolysis in the presence of certain inhibitors of cell wall synthesis. Scand. J. Infect. Dis. **7**:185–190.
27. **Shah, P. M.** 1982. Paradoxical effect of antibiotics. I. The 'Eagle effect.' J. Antimicrob. Chemother. **10**:259–260.
28. **Simard, P., and M. G. Bergeron.** 1982. Inoculum size effect on the MIC of cefoperazone, moxalactam, cefotaxime, cefoxitin and cephalothin for 118 strains of *Haemophilus influenzae* including 'tolerant' micro-organisms. J. Antimicrob. Chemother. **10**:397–402.
29. **Slater, G. J., and D. Greenwood.** 1983. Detection of penicillin tolerance in streptococci. J. Clin. Pathol. **36**:1353–1356.
30. **Sykes, R. B., A. Griffiths, and D. M. Ryan.** 1977. Comparative activity of ampicillin and cefuroxime against three types of *Haemophilus influenzae*. Antimicrob. Agents Chemother. **11**: 599–604.
31. **Syriopoulou, V. P., D. W. Scheifele, C. M. Sack, and A. L. Smith.** 1979. Effect of inoculum size on the susceptibility of *Haemophilus influenzae* b to beta-lactam antibiotics. Antimicrob. Agents Chemother. **16**:510–513.
32. **Taylor, P. C., F. D. Schoenknecht, J. C. Sherris, and E. C. Linner.** 1983. Determination of minimum bactericidal concentrations of oxacillin for *Staphylococcus aureus*: influence and significance of technical factors. Antimicrob. Agents Chemother. **23**:142–50.
33. **Turk, D. C.** 1977. A comparison of chloramphenicol and ampicillin as bactericidal agents for *Haemophilus influenzae* type b. J. Med. Microbiol. **10**:127–131.
34. **Woolfrey, B. F., M. E. Gresser-Burns, D. Ehresman, and R. T. Lally.** 1988. Characterization of β-lactam antagonist in Supplement C. Diagn. Microbiol. Infect. Dis. **10**:213–219.
35. **Woolfrey, B. F., M. E. Gresser-Burns, and R. T. Lally.** 1987. Ampicillin killing curve patterns of *Haemophilus influenzae* type b isolates by agar dilution plate count method. Antimicrob. Agents Chemother. **31**:1711–1717.
36. **Woolfrey, B. F., M. E. Gresser-Burns, and R. T. Lally.** 1988. Effect of temperature on inoculum as a potential source of error in agar dilution plate count bactericidal measurements. Antimicrob. Agents Chemother. **32**:513–517.
37. **Woolfrey, B. F., and R. T. Lally.** 1989. Tolerance to penicillin in

streptococci of viridans group. J. Clin. Pathol. **42**:1005–1006.
38. **Woolfrey, B. F., R. T. Lally, and M. N. Ederer.** 1985. Evaluation of oxacillin tolerance in *Staphylococcus aureus* using a novel method. Antimicrob. Agents Chemother. **28**:381–388.
39. **Woolfrey, B. F., R. T. Lally, and M. N. Ederer.** 1986. Influence of technical factor variations during inoculum preparation on the agar dilution plate-count method for quantitation of *Staphylococcus aureus* oxacillin persisters. Antimicrob. Agents Chemother. **30**:792–793.
40. **Yourassowsky, E., and M. P. Van Der Linden, and M. J. Lismont.** 1979. Growth curves, microscopic morphology, and subcultures of beta-lactamase-positive and -negative *Haemophilus influenzae* under the influence of ampicillin and cefamandole. Antimicrob. Agents Chemother. **15**:325–331.
41. **Yourassowsky, E., M. P. Vander Linden, M. J. Lismont, and E. Schoutens.** 1978. Qualitative study of the paradoxical zone phenomenon of penicillins against 17 bacterial species of clinical importance. Chemotherapy **24**:92–96.