Effects of Antibiotics on Induction, Viability, and Reversion Potential of L-Forms of *Haemophilus influenzae*

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L-form variants of *Haemophilus influenzae* were found to be more susceptible than their parent bacilli to streptomycin, kanamycin, tetracycline, erythromycin, and chloramphenicol if L-form inducer, penicillin, was present. This greater susceptibility was defined as the bactericidal effect for the variant forms of a concentration of antibiotic that is lower than but approximates the level bacteriostatic for the parents. In the absence of penicillin, reversion to and growth as bacilli occurred, although kanamycin, chloramphenicol, and erythromycin were lethal for round-body inocula on antibiotic reversion media in a few instances. The development of round bodies from bacillary inocula on induction medium was inhibited by bacteriostatic concentrations of chloramphenicol or tetracycline. The bacilli survived for 72 hr or more, whereas round bodies were dead within 24 hr.

A previous report from this laboratory (3) contributed to the increasing evidence (1) that microbial L-forms may play a significant role in chronic or recurrent infections. L-form variants of *Haemophilus influenzae* were found to occur in sputa from patients receiving penicillin therapy for *H. influenzae* infections of the bronchi, and recurrence of infection after cessation of therapy was related to reversion of the organism from L-form to bacillus.

Kagan et al. (2) suggested that, when antibiotics capable of inducing variant forms are used for therapy, a course of a second antibiotic should follow, since L-forms of staphylococci were found to be more sensitive to many antibiotics than were the parent bacteria. Montgomerie et al. (5) found that the use of kanamycin or erythromycin in combination with penicillin resulted in rapid elimination of both bacterial and variant populations of *Streptococcus faecalis* from rat kidneys. Other studies (6, 10, 11) on the antibiotic susceptibility of a variety of bacterial L-forms also showed the variant forms to be generally more sensitive than parent bacteria to antibiotics whose activity is not dependent upon inhibition of cell wall synthesis. However, these studies were either done on stable L-forms, whose pathogenicity remains questionable, or on nonstable variants which require high concentrations of penicillin for maintenance. This circumstance might well influence the effects of other antibiotics. Experi-

ments reported here were designed to compare the antibiotic susceptibilities of *H. influenzae* and those of its penicillin-induced variants in both the presence and the absence of penicillin. Further, and perhaps more significantly, studies were performed to determine the effects of commonly used antibiotics on the induction of variants by penicillin and on the viability of preformed variants, including their potential for reversion to bacillary form and formation of L colonies.

The terms spheroplast and protoplast are here avoided for lack of precise and consistent usage. Round body is used for the spherical or ameboid form bound by a cytoplasmic membrane that develops when bacterial cell wall synthesis is inhibited by penicillin. L-form, L variants, and round bodies are used interchangeably in referring to variant forms that revert to bacterial form in the absence of inducer and grow as L colonies in the presence of inducer. Nonreverting forms are referred to as stable L-forms.

MATERIALS AND METHODS

Media. Base medium was either Levinthal broth or agar prepared from Trypticase Soy Broth (BBL) or Trypticase soy base supplemented with yeast-hemin extract (BBL). These were equally satisfactory and gave similar results in all tests. L-form induction broth (referred to as IB) consisted of liquid medium plus 10% sucrose and 100 to 200 µg of penicillin/ml as inducer. L-form induction-maintenance agar medium (referred to as IA) was prepared from base

agar medium plus 10% sucrose and 50 μ g of penicillin/ml and was used to determine the L colony formation capabilities of various inocula. Reversion agar (RA) was identical to IA but contained no penicillin and was used to determine the ability of L-form inocula to revert to and multiply as bacilli. Chocolate-agar and Levinthal agar were also satisfactory reversion media. *H. influenzae* variant forms apparently belong to the broad group of L-forms (7) that do not require an osmotically stabilized environment for preservation of structural integrity and viability. During the course of these studies, sucrose was found to be unnecessary for the maintenance of viability and developmental potential of the *H. influenzae* variants but was used throughout for the purpose of uniformity.

Antibiotic test media were IA and RA containing 0.5, 1.0, 5, 10, 50, and 100 μ g (per ml) of streptomycin, kanamycin, tetracycline, chloramphenicol, or erythromycin and were used to determine the effects of these antibiotics on L colony formation and reversion capabilities of variant forms.

Organisms and inocula. All strains of *H. influenzae* studied were isolated from patients with chronic bronchitis hospitalized at the Veterans Administration Hospital in Madison, Wis., and were selected to include strains resistant to streptomycin, tetracycline, and erythromycin. The bacteriostatic and bactericidal levels of each antibiotic for each strain were determined by standard tube dilution technique in Levinthal broth with appropriate subcultures to Levinthal or yeast-hemin-agar medium after 18 to 24 hr of incubation.

Round-body inocula (RBI) were produced by adding 100 to 200 μ g of penicillin per ml to actively growing (4 to 6 hr) bacillary cultures in IB. Conversion to round bodies, determined by microscopic examination of wet mounts, was usually complete within 6 hr, but growth and enlargement continued for 48 to 72 hr. L colony inocula were agar blocks of L colonies that had developed in 5 to 7 days on IA inoculated with either RBI or bacillary cultures in Levinthal broth. All bacillary inocula were actively growing 6- to 18-hr broth cultures.

Experimental procedures. RBI were plated to IA, RA, IA antibiotic test medium, and RA antibiotic test medium at 8, 24, 48, and 72 hr after addition of penicillin to determine the antibiotic susceptibilities of round bodies of various ages. These media were also inoculated with samples of each RBI to which penicillinase (Penase concentrate; Difco) was added in order to observe development of the inocula in the absence of any active penicillin, whether carried over in the inoculum or bound to the cytoplasmic membranes (7) of the round bodies.

For comparing effects of the test antibiotics on variant forms with those on parent bacilli and for determining the effects of simultaneous exposure to test antibiotic and inducer on the conversion from parent to L-form, the 4- to 6-hr bacillary cultures intended for RBI were plated on the various media immediately before addition of the inducer, penicillin.

Inoculations were performed by carefully streaking one loopful (loop 2 mm in diameter) of inoculum

over the surface of agar media. Incubation was at 37 C in a CO₂ incubator and observations of the plates were made both visually and microscopically (10 $\times$ objective) after 8, 24, and 48 hr and at the end of incubation (7 to 10 days). The viability of both round-body and bacillary inocula on all test media was determined by agar block transfers to RA plus penicillinase after 4 to 6 hr of incubation, and again after 24 and 48 hr if growth was not grossly visible. To determine whether continued growth and multiplication in the variant state would alter the subsequent behavior of RBI on antibiotic test media, the studies were repeated with round bodies derived from L colonies by using the agar block transfer technique for inoculation of test media.

RESULTS

Development on IA and RA. During the first 18 hr of incubation on IA, round bodies increased in size and multiplied into clusters or dense centers (Fig. 1a) which developed into small L colonies (Fig. 1b) by 36 hr. Figure 1c shows typical mature L colonies after 7 days of incubation. Fewer than 25% of the round bodies develop into L colonies, and no increase was noted when Mg, yeast extract, or horse serum was added to IA. When the RBI were plated to RA, 80% or more reverted to bacillary form and produced almost confluent bacillary growth in 24 hr. Addition of penicillinase to the inocula before plating allowed reversion of almost all of the round bodies. A few nonviable forms always could be seen scattered between colonies before bacillary growth became confluent and obscured the residual inoculum. These results indicate that such penicillin-induced RBI consist of a mixed population. Few of the round bodies have the capacity for growth and multiplication as L-forms, many are able to repair the penicillin-induced cell wall damage and revert in spite of membrane-bound or carryover penicillin, a few more revert if the membrane-bound penicillin is destroyed by penicillinase, and a few are not viable (Table 1). Results were similar with round bodies from 8 to 72 hr old. Most L colonies, however, were produced from 24- and 48-hr inocula, more nonviable forms being present at 72 hr.

RBI from L colonies behaved similarly and therefore must also consist of a population with varied growth potential. Figure 2a shows the dense agar-imbedded centers of L colonies stripped of peripheral growth by the agar block transfer technique. Figure 2b shows round bodies deposited from the peripheries of the L colonies as the agar block was pushed across fresh IA and shows scattered young L colonies and beginning dense centers developing from some of the round bodies at 36 hr. Figure 2c shows new peripheral growth around the stripped colonies of the agar

FIG. 1. Development of round-body inocula on induction agar. (a) Enlarged round bodies in clusters with beginning dense centers (arrows) after 18 hr of incubation. $\times 100$. (b) Small L colonies after 36 hr of incubation. $\times 100$. (c) Mature L colonies after 7 days of incubation. $\times 100$.

TABLE 1. *Growth potential of round-body inocula (RBI)^a*

Inoculum	Growth on IA media	Growth on RA media
RBI	1 + (L colonies)	2-3 + (bacillary)
RBI + penicillinase	4 + (bacillary)	3-4 + (bacillary)

^a Growth defined as: 1 +, scattered colonies; 2 +, numerous colonies; 3 +, almost confluent growth; 4 +, confluent growth.

block inoculum after 36 hr on IA. (Microscopic examination of the agar block for new peripheral growth provides a reliable means of checking the viability of the L colony inocula.)

Bacillary inocula on IA developed into round bodies, dense centers, and mature colonies in sequence similar to that of RBI. On RA, confluent bacillary growth occurred.

Antibiotic susceptibility of round-body and L colony inocula. Round-body and L colony inocula were more sensitive to streptomycin, kanamycin, chloramphenicol, tetracycline, and erythromycin than were parent bacilli when plated to antibiotic test IA. No significant differences were noted in the results obtained with L colony inocula or with 8-, 24-, 48-, or 72-hr broth-induced round bodies.

A concentration of antibiotic slightly lower than the minimal inhibitory concentration of that drug for bacillary parents, as determined by tube dilution tests, completely inhibited development of the round bodies into L colonies on IA, was bactericidal for most of the variant forms after 5 hr (no growth or only few colonies appearing on subculture to penicillinase RA), and was completely bactericidal within 24 hr. Bactericidal and bacteriostatic levels of the antibiotics for two representative strains of *H. influenza* and the bactericidal levels for their L-form variants are presented in Table 2. Four other strains of *H. influenzae* and their derived variants responded as did the sensitive strain (Table 2), variants from two others required 10 μ g of erythromycin per ml in IA for inhibition of L colony growth, and variants from one other strain were inhibited by 0.5 μ g of chloramphenicol per ml.

However, when RBI were inoculated to antibiotic RA or if penicillinase was added to the inoculum before plating to IA, reversions to bacillary state and growth occurred at concentrations of antibiotic lethal for RBI in the presence of penicillin. Results of plating RBI to antibiotic test RA were usually comparable to results obtained on IA if penicillinase was added before plating and reflected the inhibitory patterns of

bacillary inocula on antibiotic test RA. That is, concentrations of antibiotic in RA which did not inhibit growth of parent bacillary forms did not usually inhibit reversion and growth of the variants on RA but were lethal for RBI on IA unless penicillinase was added.

Some differences were apparent. Reversion of RBI with subsequent bacillary growth was often delayed 12 to 18 hr and occurred from fewer of the variant forms on antibiotic test RA than on RA alone. When penicillinase was added to the RBI, reversion on antibiotic RA occurred earlier and from many more of the variant forms, although growth was not as heavy as that on RA. These effects were particularly noticeable with kanamycin, chloramphenicol, and erythromycin. RB inoculated to relatively low concentrations (1, 1, and 5 μ g/ml, respectively) of these antibiotics in RA either failed to revert and were not viable after 24 hr or showed delayed and minimal bacillary growth in 38 hr. When penicillinase was added to the RBI before plating to these antibiotic media, growth occurred but was less heavy than growth from the same inocula on RA.

RB inoculated on IA containing lethal concentrations of streptomycin and kanamycin seemed to shrink before losing viability in 18 to 24 hr, whereas inoculations on lethal concentrations of chloramphenicol, tetracycline, and erythromycin resulted in swelling, loss of density, and apparent fusion of the round bodies.

Effects of penicillin concentration. The use of 10 rather than 50 μ g of penicillin per ml in antibiotic IA showed that penicillin concentration may affect results obtained with RB inocula. Significant differences were not noted with kanamycin, chloramphenicol, and erythromycin; however, with streptomycin and tetracycline, higher concentrations were required for expression of lethal activity against RB on IA containing 10 μ g of penicillin per ml than were required with 50 μ g of penicillin per ml. For example, RBI from a streptomycin-resistant strain were able to develop into L colonies in medium containing (per ml) 50 μ g of streptomycin and 10 μ g of penicillin but not in medium containing 50 μ g of streptomycin and 50 μ g of penicillin. Variants from another strain developed into L colonies on medium containing (per ml) 1 μ g of tetracycline and 10 μ g of penicillin, but not in the presence of 1 μ g of tetracycline and 50 μ g of penicillin; 5 μ g of tetracycline was, however, lethal in the presence of 10 μ g of penicillin.

Simultaneous exposure of bacillary forms to test antibiotic and L-form inducer. The morphological character of the inoculum, that is, whether plated as bacillary parent or round body, had little effect on the ultimate response to antibiotics in IA. At

FIG. 2. (a) Residual dense centers of *L* colonies stripped of peripheral round bodies by agar block transfer. $\times 100$. (b) Round bodies deposited on penicillin-agar from the peripheries of *L* colonies during agar block transfer after 36 hr of incubation showing small *L* colonies and beginning dense centers (arrows). $\times 100$. (c) New peripheral growth around dense centers denuded as in (a) after 36 hr of incubation, indicating viability of the agar block inocula. $\times 160$.

TABLE 2. Antibiotic susceptibility of two strains of *H. influenzae* and their variant forms

Antibiotic	Sensitive strain susceptibility ($\mu\text{g/ml}$)			Resistant strain susceptibility ($\mu\text{g/ml}$)		
	Bacillary parent		L-form variant	Bacillary parent		L-form variant
	Bacteriostatic level ^a	Bactericidal level ^a	Bactericidal level ^b	Bacteriostatic level ^a	Bactericidal level ^a	Bactericidal level ^b
Streptomycin	1.56	6.25	1.0	>100	>100	50.0
Kanamycin	1.0	5.0	1.0	1.0	5.0	<1.0
Chloramphenicol	1.56	25.0	1.0	6.25	50.0	5.0
Tetracycline	1.56	50.0	1.0	6.25	50.0	5.0
Erythromycin	6.25	25.0	5.0	100	100	50.0

^a Determined by 24-hr tube dilution tests.^b Determined 24 hr after plating variant forms to antibiotic test induction agar.

concentrations of drug that allowed growth of L colonies from RBI, bacillary inocula converted to round bodies and grew as L variants. Concentrations of streptomycin and kanamycin that allowed bacillary growth on RA but were lethal for round bodies on IA were also lethal for bacillary inocula on IA in 24 hr, although most bacilli first de-developed into round bodies. At concentrations that inhibited bacillary growth on RA, the bacillary inocula remained viable for 48 hr; on IA, however, viability was lost within 24 hr, although bacilli did not develop into round bodies. Results with erythromycin were similar, but death of bacillary inocula in the presence of penicillin was slower, requiring 48 hr, whereas round bodies were killed in 24 hr. Lethality of either tetracycline or chloramphenicol for bacillary inocula in the presence of penicillin was dependent upon the concentration of the drug. Concentrations of these antibiotics lethal for RBI on IA, although still permitting complete conversion of bacillary inoculum to round bodies on IA, were lethal for the bacillary inoculum within 24 hr. Concentrations that showed minimal inhibition of bacillary growth on RA (usually 1 or 5 μg of chloramphenicol and 5 μg of tetracycline per ml) and inhibited complete conversion of bacilli to round bodies on IA (residual bacillary inocula could be identified by microscopic examination of the plates) were not bactericidal on IA within 48 hr, growth occurring on subculture after 24 and 48 hr of drug exposure. RBI were killed within 24 hr by these same concentrations of chloramphenicol and tetracycline in IA. Thus, antibiotic antagonism occurred between these bacteriostatic drugs and penicillin at concentrations that halted protein synthesis necessary for round body formation but that were not bactericidal for the bacilli.

DISCUSSION

Our data on antibiotic susceptibility of L-forms of *H. influenzae* support the general supposition based on studies with other bacteria (2, 5, 6, 10, 11) that L-form variants are generally more sensitive than are their parents to antibiotics whose activity is not primarily dependent upon inhibition of cell wall synthesis. Further, our data define this greater susceptibility as the bactericidal effect on L variants of a concentration of antibiotic that is slightly lower than but approximates the minimal inhibitory concentration (bacteriostatic level) of the drug for the parent bacteria. However, unless L-form inducer, penicillin in this case, is present, cell metabolism and protein synthesis continue at subinhibitory concentrations of antibiotic, cell wall is produced, and multiplication in bacillary form ensues. We observed that membrane-bound penicillin was able to maintain adequate inhibition of cell wall synthesis on inducer-free media to allow killing of all or most of the RBI in several instances, particularly with kanamycin, chloramphenicol, and erythromycin. That penicillin was involved was apparent from results with penicillinase-treated RBI which were able to revert to bacillary form on antibiotic test media. That the penicillin was membrane-bound penicillin and not carried over in the inoculum seems most probable, for the quantity transferred (less than 1 or 2 μg) was further diluted by diffusion into the agar.

Although Montgomerie et al. (6) reported that the sensitivity of *Streptococcus faecalis* protoplasts was unaffected by presence or absence of penicillin, the relationship was not thoroughly explored. They did find that differences in susceptibility between the parent and variant forms were less marked or nearly absent

with tetracycline, polymyxin, colistin, and ristocetin on salt-containing medium. The variants on this penicillin-free medium were nonreverting or stabilized L-forms. Others (2, 9, 11) have also observed that stable L-forms and their parent forms do not show marked differences in antibiotic susceptibility. This may well reflect differences in structure and composition between the cytoplasmic membranes of L-form variants and stable L-forms. Panos (1, 7) has shown that membranes of stable L-forms contain more fatty acid and complex lipid than do nonstable forms and suggests that this strengthening of the membrane may protect stable forms against various injuries, biochemical and physical, to which nonstable forms, or protoplasts, are vulnerable.

Synergistic effects of various antibiotics in combination with penicillin are well documented (4, 8) and were demonstrated here with streptomycin and kanamycin against bacillary inoculum on IA. However, a complete understanding of the responsible mechanisms and of the greater sensitivity of stable variants to some antibiotics awaits further study. Furthermore, the reason why lethality of kanamycin, chloramphenicol, and erythromycin on RBI was independent of penicillin concentration and was demonstrable against RBI on penicillin-free media is obscure and also awaits further study. This might be explained either on the basis that, with loss of cell wall and penicillin-induced membrane damage, these antibiotics can enter the cell in adequate concentration for inhibition of protein synthesis before new cell wall can be reformed, or on the basis that the site of primary activity may be the membrane per se, exposed but not seriously damaged by low levels of penicillin. Streptomycin and tetracycline may be unable to contribute to membrane damage unless the membrane is first damaged by high levels of penicillin.

The results of these *in vitro* studies support the

concept that a second antibiotic should be added to a therapeutic regimen employing an antibiotic such as penicillin or cephalothin which can induce L-form variants of bacteria. Further, these results suggest that, for maximal expression of the bactericidal effect of the drug combination and for avoidance of possible antagonistic effects, the L-form inducer should be administered before addition of the second antibiotic.

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