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# Microbiological Characterization of Everninomicins B and D

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Everninomicins B and D are components of a complex of antibiotic substances produced by *Micromonospora*. Both were shown to be highly active inhibitors of growth of all gram-positive bacteria, *Neisseria*, and *Bacteroides* studied in vitro. Potency of activity appeared to be greater than that of chloramphenicol, but less than that of penicillin G, when assayed against strains susceptible to each of the drugs. The everninomicins were bacteriostatic for all strains tested, except group A streptococci. No facultatively anaerobic gram-negative bacilli were susceptible. Resistant mutants were selected with difficulty from susceptible staphylococci in the laboratory, and these demonstrated no cross-resistance to available antimicrobial agents. Most variations in media, growth conditions, or procedure of assay had little or no effect on antimicrobial activity. Only addition of serum or increase in inoculum size reduced antibacterial activity. Significant differences in activity of the two components were encountered infrequently; the B component was four- to sixfold more active against gonococci and group A streptococci, whereas the D component was fourfold more active against enterococci. Because of the high degree of in vitro activity and lack of resistance among susceptible genera of bacteria, the everninomicins clearly merit further careful study as potential therapeutic agents.

Everninomicin is a complex of at least five related antibiotic substances produced by *Micromonospora carbonacea* (9, 15). Several components appear to contain dichloroisoeverninic acid in combination with a polysaccharide moiety (6, 7). One component, everninomicin D, has been shown to inhibit growth of gram-positive organisms (16) and meningococci (11) in vitro and to be efficacious in treatment of experimentally infected mice. It was also remarkably free of acute toxicity in experimental animals (2, 16).

The present study employed two components of the complex, everninomicins B and D. It was designed to assess the following characteristics of the two drugs: (i) spectrum and relative potency of activity against a wide range of clinical isolates; (ii) qualitative nature of the antibiotic activity; (iii) effect of variations in growth media, method of assay, and inoculum size on activity; and (iv) effect of serum upon antimicrobial activity. In addition, the characteristics of resistance to the everninomicins and degree of cross-resistance to currently available antibacterial agents were assessed among pyogenic bacteria.

## MATERIALS AND METHODS

**Antibiotics.** The antibiotics and the sources from which they were obtained were: everninomicins B and D (Schering Laboratories, Bloomfield, N.J.), chloramphenicol (Parke, Davis & Co., Detroit, Mich.), penicillin G (Chas. Pfizer & Co., Inc., New York), tetracycline hydrochloride (Roerig Division, Pfizer Inc., New York), erythromycin (Abbott Laboratories, North Chicago, Ill.), lincomycin (Upjohn Co., Kalamazoo, Mich.), and rifampin (Dow Chemical Co., Indianapolis, Ind.). Stock solutions of everninomicin B and D were prepared at least every third day by dissolving 0.005 g of the powder in as little 0.1 N NaOH as possible (usually 0.10 to 0.15 ml), followed by addition of sufficient distilled water to yield a final concentration of 200 to 400 μg/ml. These were sterilized by filtration and stored at 4 C. Immediately before use, a final dilution was made into the appropriate medium for assay of antibacterial activity. Neither everninomicin B or D in concentrations of 100 μg/ml or less altered the pH of the growth media employed for assay of antibacterial activity.

**Microorganisms.** Most of the microorganisms employed were recently isolated from clinical specimens (Diagnostic Microbiology Laboratories of the Shands Teaching Hospital, Gainesville, Fla; Creighton Memorial St. Josephs Hospital, Omaha, Neb.; and the Central Laboratory, W. T. Edwards Hospital, Tampa, Fla.). Strains were often selected because of known resistance to commonly available antimicrobial agents. Five strains of *Staphylococcus aureus* that were highly resistant to methicillin and cephalothin were kindly supplied by John V. Bennett, National Center for Disease Control, Atlanta, Ga. Identity of microorganisms was confirmed by standard morphological, cultural, biochemical, and serological techniques (4, 5).

**Assays of antibacterial activity.** Antibacterial activity was assayed in broth or agar cultures to which serial twofold dilutions of the drugs had been added. Minimal inhibitory concentration (MIC) was the lowest concentration of drug in which there was no visible growth after incubation for 18 h at 37 C in 10% $CO_2$ in air. Subcultures to antibiotic-free medium were made of portions (0.01 ml) from each of the clear tubes in the broth dilution assays; minimal bactericidal concentration (MBC) was the lowest concentration of drug that prevented growth in these subcultures. Assays were performed with brain heart infusion broth (BHIB; BBL, Cockeysville, Md.) or agar, unless otherwise indicated. Inocula were prepared from overnight cultures and adjusted to yield $10^5$ to $10^6$ viable units per ml of broth medium or per $cm^2$ of agar (applied by a 0.01-ml inoculating loop). Susceptibility of microorganisms to most commonly available antimicrobial agents was determined by an identical method, or disc diffusion (1), or both. Methods of procedure for susceptibility testing of mycobacteria have been described in detail previously (11).

## RESULTS

**Spectrum and potency of activity.** Results obtained in many assays with the two everninomicins were very similar or identical. Thus results will be presented for everninomicin B only, unless significant differences were noted in the activity of the two drugs.

The cumulative percent of strains of pyogenic cocci (streptococci, pneumococci, gonococci, meningococci, and staphylococci) inhibited by the various concentrations of everninomicins B and D are shown in Fig. 1. All strains were inhibited by 4.0 μg or less of each of the drugs per ml.

The activities of everninomicin B, chloramphenicol, and penicillin G assayed against 43 strains of *Neisseria meningitidis* in Mueller-Hinton broth (Difco) are compared in Fig. 2.

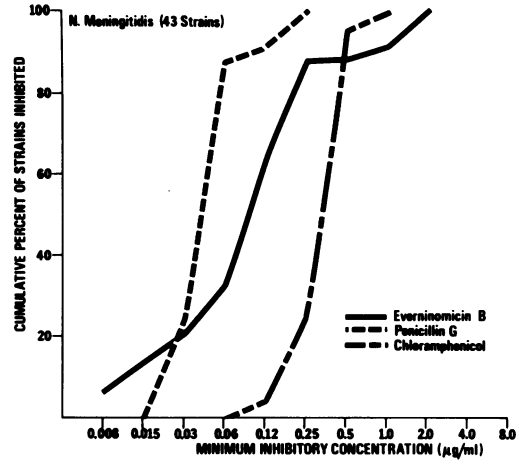


FIG. 2. *Comparison of activities of everninomicin B, chloramphenicol, and penicillin G against Neisseria meningitidis in Mueller-Hinton broth.*

The potency of everninomicin B was intermediate between that of penicillin G (greatest) and chloramphenicol (least). This relationship was also observed with other pyogenic cocci that were susceptible to each of the three drugs. Everninomicins B and D were equally potent against strains of meningococci that were susceptible or resistant to the sulfonamides and rifampin (not shown).

Both everninomicins were highly active inhibitors of growth of strains of *Neisseria gonorrhea* when assayed in Mueller-Hinton agar plus chocolatized blood (Fig. 3). The B component appeared to be severalfold more active; however, fewer strains were tested with this drug.

Everninomicin B inhibited each of 28 strains of *Staphylococcus aureus* in concentrations of 0.25 to 2.0 μg/ml (Fig. 4). Results with the D

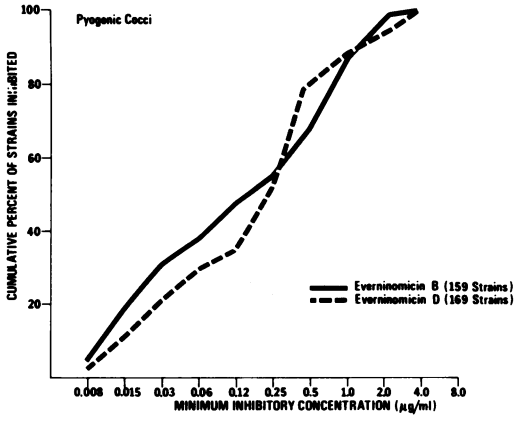


FIG. 1. *Activity of everninomicins B and D against pyogenic cocci (streptococci, pneumococci, gonococci, meningococci, and staphylococci) in vitro.*

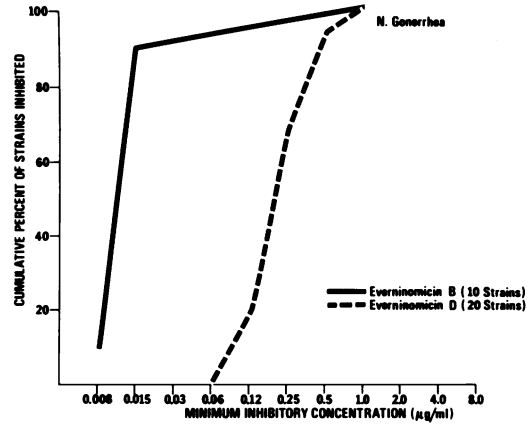


FIG. 3. *Activity of everninomicins B and D against Neisseria gonorrhoeae in vitro.*

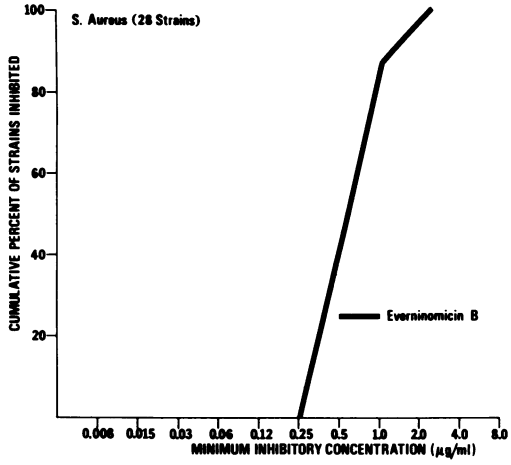


FIG. 4. *Activity of everninomicin B against S. aureus in brain heart infusion broth. Five strains were highly resistant to methicillin and cephalothin.*

component were nearly identical. It is noteworthy that both drugs inhibited the five strains that were highly resistant to penicillin G, methicillin, cephalothin, erythromycin, lincomycin, streptomycin, and tetracycline. Similar results were obtained when both drugs were tested against 20 strains of *Staphylococcus epidermidis*; MIC ranged for 0.015 to 1.0 μg/ml (not shown).

The activity of the everninomicins against streptococci and pneumococci is summarized in Fig. 5 through 8. Everninomicin B was severalfold more active against group A streptococci; all 20 strains were inhibited by 0.03 μg or less of

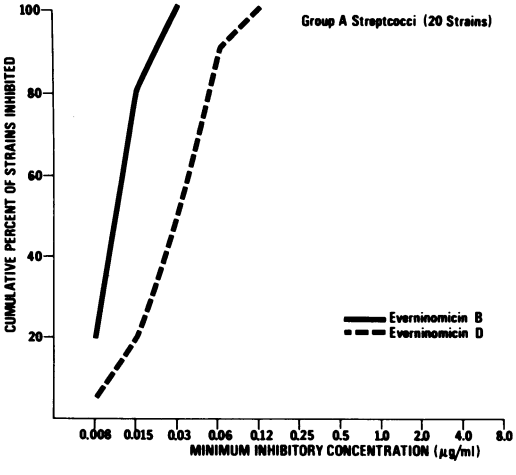


FIG. 5. *Activity of everninomicins B and D against group A streptococci in vitro. Four strains were resistant to tetracycline, erythromycin, and lincomycin.*

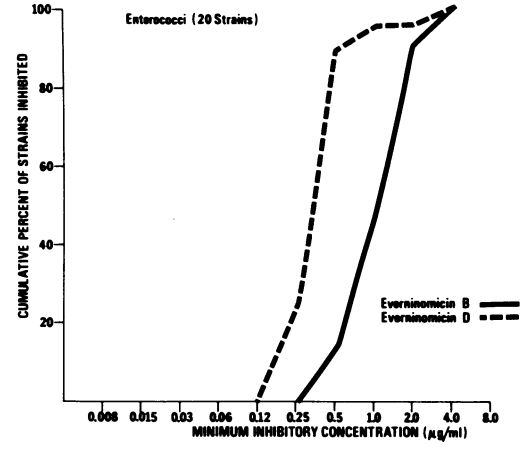


FIG. 6. *Susceptibility of enterococci to everninomicins B and D in brain heart infusion broth.*

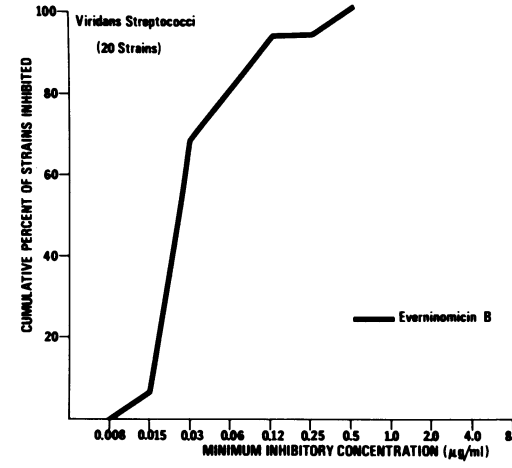


FIG. 7. *Activity of everninomicin B against viridans streptococci in brain heart infusion broth.*

the drug per ml. Four of the strains of group A streptococci were highly resistant (12) to erythromycin, lincomycin, and tetracycline (MIC in excess of 32 μg/ml). When assayed against 20 strains of enterococci, everninomicin D was found to be severalfold more potent; however, both drugs inhibited all strains at a concentration of 4.0 μg or less per ml. The two drugs were equally active against 20 strains of viridans streptococci; MIC ranged from 0.015 to 0.5 μg/ml. Each of 20 strains of pneumococci were lighly susceptible to both everninomicins B and D (MIC 0.06 μg/ml or less).

Each of 83 isolates representing 11 genera of gram-negative bacilli (*Acinetobacter, Enterobacter, Klebsiella, Providencia, Serratia, Shigella, Escherichia, Citrobacter,* Alkales-

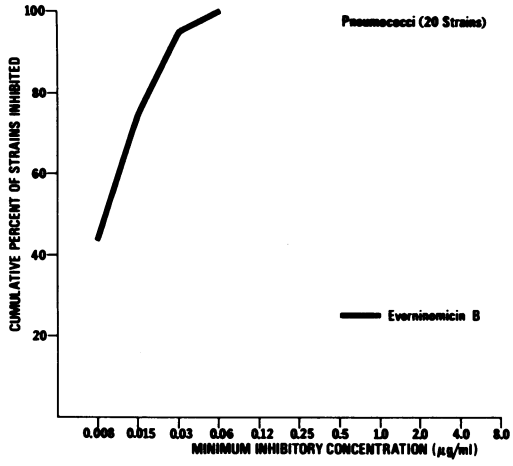


FIG. 8. *Activity of everninomicin B against pneumococci in brain heart infusion broth.*

cens-dispar, *Pseudomonas*, *Salmonella*, and *Proteus*) were found to be resistant to at least 20 μg of both everninomicins B and D per ml when grown aerobically.

The activity of everninomicin B was assayed against three strains of *Bacteroides* and three strains of *Clostridium* in BHIB in an anaerobic environment (Anaerobic chamber, type B, Coy Manufacturing Co., Ann Arbor, Mich.); each of the isolates was inhibited by 1.0 μg or less per ml (Table 1). Each of four isolates of *Haemophilus influenzae* was inhibited by 8.0 μg of everninomicin B per ml when assayed in HIB supplemented with hemoglobin and IsoVitalex (BBL) (Table 2).

Activity of everninomicin B was determined against five isolates of mycobacteria in Proskauer and Beck liquid medium (Table 1). The drug was either inactive or weakly tuberculostatic in concentrations of 50 to 100 μg/ml.

**Qualitative nature of antibiotic activity.** Subcultures from each clear tube in all broth dilution assays yielded viable microorganisms, except for those performed for group A streptococci in which MIC always equalled MBC. This suggested that the everninomicins were bactericidal for group A streptococci and bacteriostatic for other susceptible microorganisms. To substantiate this difference, the effects of everninomicin B, lincomycin, and penicillin G on survival of susceptible bacteria in liquid medium (BHIB) were assayed. In these experiments, approximately $10^4$ viable units per ml of pneumococci or group A streptococci from logarithmic phase cultures was exposed to each of the three drugs in concentrations eightfold in excess of their respective MIC. Duplicate portions were removed from each tube after 2, 4, 6, and 8 h of incubation, and surviving organisms were enumerated by the standard plate dilution method (Fig. 9 and 10). Both strains grew well in the absence of drugs. Penicillin G resulted in death of viable cells within 8 h. Lincomycin merely prevented growth or induced slight diminution of viable organisms in each assay. Everninomicin B was clearly bacteriostatic for the pneumococcus; however, it was bactericidal

TABLE 1. *Activity of everninomicin B Against Bacteroides, Clostridium, Haemophilus influenzae, and Mycobacterium in vitro*

| Organism tested | Medium | MIC (μg/ml) |
|---|---|---|
| Anaerobic bacteria | | |
| *Clostridium perfringens* | Brain heart infusion broth | ^.03 |
| *C. sporogenes* | | 0.008 |
| *C. histolyticum* | | 0.008 |
| *Bacteroides fragilis* | | 0.008 |
| *B. oralis* | | 1.0 |
| *B. nucleatum* | | 1.0 |
| Mycobacteria | | |
| *M. tuberculosis*, H37Rv | Proskauer and Beck liquid | 50.0 |
| *M. kansasii* | | 50.0 |
| *M. scrofulaceum* | | 50.0 |
| *M. intracellulare* | | 100.0 |
| *M. fortuitum* | | >100.0 |
| *Haemophilus influenzae* | | |
| Strain 1, serotype b | Heart infusion, plus hemoglobin and IsoVitalex | 8.0 |
| Strain 2, serotype e | | 8.0 |
| Strain 3, nontypable | | 8.0 |
| Strain 4, nontypable | | 8.0 |

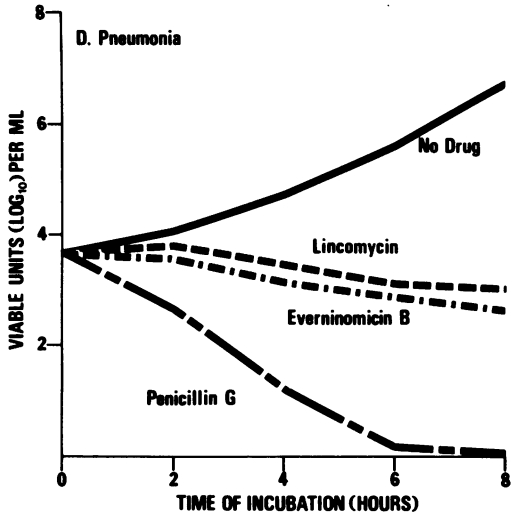


FIG. 9. *Survival of D. pneumonia in brain heart infusion broth with added penicillin G, lincomycin, or everninomicin B.*

for the group A streptococcus with killing kinetics closely approximating those of penicillin G.

**Effects of variations in media, method of assay, and inoculum size.** The effect of use of broth media other than BHIB on results of assays of activity of the everninomicins was assessed. Equivalent MIC were obtained when five strains of group A streptococci were tested in each of four additional broths: Todd-Hewitt (BBL), antibiotic medium no. 3 (Difco), heart infusion (Difco), and Trypticase soy (BBL). Equivalent MIC were found when five strains of *S. aureus* were tested in Todd-Hewitt (BBL), Mueller-Hinton (BBL), heart infusion (Difco), Trypticase soy (BBL), and nutrient (BBL) broths; however, MIC were fourfold or more lower for each strain when the assay was performed in antibiotic medium no. 3 (Difco).

Results of assays of activity of the two drugs against 43 strains of meningococci were similar when performed by the broth-dilution (Mueller-Hinton) and agar-dilution (Mueller-Hinton) techniques. Results were also similar when assays using *S. aureus* (five strains) were (i) incubated in air, 10% $CO_2$ in air, or anaerobically and (ii) read at 18, 24, or 48 h.

The effect of variation of inoculum size on activity of everninomicin B was determined (Table 2). MIC of the drug for three strains of group A streptococci and two strains of *S. aureus* fell progressively as the test population was decreased in 10-fold steps from $10^6$ to $10^3$ viable units per ml.

**Resistance to the everninomicins.** Most of the microorganisms employed in this study were selected because of known resistance to commonly available antimicrobial agents. All gram-positive bacteria and *Neisseria* tested were susceptible to 4.0 μg or less of the everninomicins per ml, and no "skipped tubes" were noted in the dilution assays. Each of the components of the complex was equally active against organisms known to be suceptible or resistant to penicillin G, methicillin, cephalothin, chloramphenicol, erythromycin, lincomycin, streptomycin, sulfadiazine, tetracycline, vancomycin, rifampin, polymyxin B, and gentamicin.

Since no naturally occurring resistance to the everninomicins was detected among pyogenic cocci, an attempt was made to select for resistant mutants among a large population of susceptible cells. Eight strains of *S. aureus* were chosen for study; each was initially susceptible to the everninomicins, penicillin G, cephalothin, chloramphenicol, erythromycin, lincomycin, tetracycline, rifampin, and gentamicin in tube dilution assays. Approximately $10^8$ cells of each strain per ml was then serially transferred daily into increasing concentrations (twofold increments) of everninomicin D in BHIB. Resistance appeared in a slow, stepwise fashion in each instance. After 6 to 18 serial transfers, organisms were recoverable that grew in 32 μg of everninomicin D per ml. These resistant cells grew more slowly, formed smaller colonies, and produced less pigment and hemolysis on blood

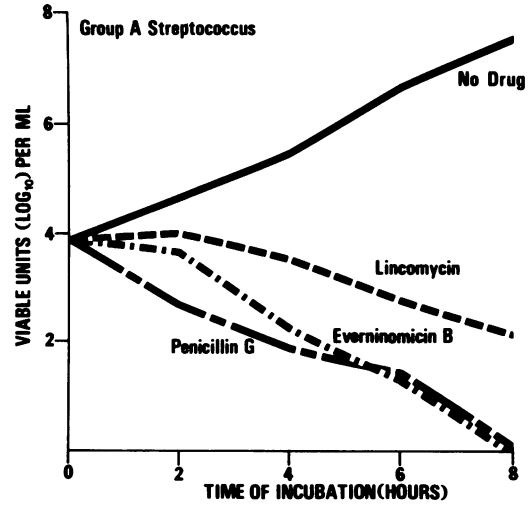


FIG. 10. *Survival of a strain of group A streptococcus in brain heart infusion broth with added penicillin G, lincomycin, or everninomicin B.*

TABLE 2. *Effect of variations in inoculum size on activity of everninomicin B in brain heart infusion broth*

| Organism | Strain no. | MIC (μg/ml) | | | |
|---|---|---|---|---|---|
| | | $10^{6}$ [a] | $10^{5}$ | $10^{4}$ | $10^{3}$ |
| *S. aureus* | 7 | 0.5 | 0.25 | 0.03 | 0.01 |
| *S. aureus* | 8 | 0.5 | 0.12 | 0.03 | 0.01 |
| Group A strep | 2 | 0.03 | 0.01 | 0.004 | 0.002 |
| Group A strep | 3 | 0.12 | 0.01 | 0.002 | 0.002 |
| Group A strep | 4 | 0.12 | 0.03 | 0.004 | 0.002 |

[a] Viable units per milliliter.

agar than the parent cells from which they were derived. Each of the progeny was found to be equally resistant to everninomicin B in dilution assays. However, these resistant strains retained equal susceptibility to the other chemically unrelated antimicrobials. Most of these resistant cells reverted to susceptibility to the everninomicins following three to six serial transfers on enriched, antibiotic-free medium.

**Effect of serum upon antimicrobial activity.** The effect of heat-inactivated, sterile human and horse sera on the antimicrobial activity of everninomicin B was assayed in duplicate with four strains of *S. aureus* by the method reported by Kirby et al. (8). Calculation of the percentage of antibiotic bound was based upon the MIC determined in BHIB and in BHIB plus 50% serum. Results were identical for each of the strains and indicated that the drug was 95.5% bound in both sera.

## DISCUSSION

Both everninomicins B and D proved to be potent inhibitors of growth of all pyogenic cocci tested. MIC ranged from 4.0 to 0.001 μg/ml; concentrations of 0.25 μg/ml and above were required to inhibit staphylococci and enterococci, whereas levels of 0.125 μg/ml or less were effective against all group A streptococci, gonococci, and pneumococci. In general, the potency of both drugs was greater than chloramphenicol and less than penicillin G. The MIC were comparable to or slightly less than those of erythromycin and four- to tenfold less than those of lincomycin when obtained by a similar method (5, 10, 12). Everninomicin B was active against strains of *Clostridium* and *Bacteroides*, inhibited each of four strains of *H. influenzae* at 8.0 μg/ml, and was inactive or weakly tuberculostatic in concentrations of 50 to 100 μg/ml.

Differences between the two components of everninomicin were minor. The B component appeared to be severalfold more active against gonococci and group A streptococci, whereas the D component was approximately fourfold more active against enterococci. Other differences were slight and within the limits of the error of the dilution methods.

Variations in methodology and conditions of testing, with a few exceptions, had little effect upon activity of the everninomicins. MIC for most organisms were uniform when determined by agar or tube dilution, in air, in 10% $CO_2$ in air, or anaerobically. Results were comparable when assays were performed simultaneously in a variety of growth media commonly used for antimicrobial susceptibility testing; an exception was the reduction of MIC for strains of *S. aureus* by fourfold in antibiotic medium no. 3. The most profound effects on the activity of the everninomicins were induced by changes in the size of the bacterial inoculum or by addition of serum. MIC of everninomicin B increased an average of threefold for each tenfold increase in bacterial inoculum over the range of $10^{3}$ to $10^{6}$ viable units of *S. aureus* or group A streptococci per ml in the assay medium. The binding of everninomicin B by human and horse serum was calculated to be 95.5%. The binding of the D component by human serum, assayed by an identical method, has been reported to be 94% (16).

Results to date clearly indicate that the everninomicins are unique, both chemically (6, 7) and microbiologically (16). No naturally occurring resistance has been detected among gram-positive bacteria or *Neisseria* and no cross-resistance with commonly available antimicrobial agents has been demonstrated among resistant mutants selected in the laboratory. These observations suggest that the mechanism of action of the everninomicins may be different from that of available drugs. In addition, the two drugs were found to be bacteriostatic for all susceptible strains, except group A streptococci. The bactericidal activity against group A streptococci was suggested by observation of equivalent MIC and MBC in broth dilution assays and was confirmed by quantitative studies of the killing kinetics. This qualita-

tive difference in antibacterial activity for closely related species has been noted previously only when concentrations of bacteriostatic drugs, considerably in excess of MIC, have been employed (3, 10, 14, 17).

The everninomicins are highly attractive as possible therapeutic agents. The most compelling reasons for further study are: (i) potency of activity against gram-positive bacteria, *Neisseria*, and strains of *Bacteroides*, (ii) lack of naturally occurring resistance among susceptible genera of bacteria, (iii) the difficulty encountered in selecting for resistant mutants and maintaining them subsequently in antibiotic-free media, (iv) lack of cross-resistance of the mutants to currently available antimicrobials, (v) the implication of a unique mechanism of antibacterial activity, and (vi) relative lack of toxicity in experimental animals (2, 16). The only possible limitations detected to date are (i) the high degree of binding by serum and (ii) a progressive diminution of antibacterial activity with increases in the inoculum size.

## ACKNOWLEDGMENTS

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