Susceptibility of *Nocardia asteroides* to 45 Antimicrobial Agents In Vitro

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A method for preparing uniformly dispersed cultures of *Nocardia asteroides* for use in tests for susceptibility to antimicrobial agents is described. The minimal inhibiting concentration (MIC) of 45 agents for cultures thus prepared was determined with the use of a replica-inoculating apparatus. Minocycline at a concentration of 3.1 µg or less/ml inhibited 90% of the strains tested, and all were inhibited by 6.3 µg/ml. An erythromycin concentration of 0.8 µg or less/ml inhibited 40% of the strains, but the MIC for most of the others was > 100 µg/ml. The other agents were generally less active. Chemically related analogues varied in activity to different degrees. Also, the MIC of each antibiotic against different strains generally varied over a wide range. Sulfonamides and trimethoprim were not active against most strains in the method used. The size of the inoculum markedly affected the MIC of sulfonamides and had variable effects on other agents. Marked synergy of erythromycin with ampicillin was demonstrated for nearly all strains tested.

*Nocardia asteroides* is one of the opportunistic pathogens which are being encountered with increasing frequency in infections of patients under prolonged treatment with corticosteroids and immunosuppressive agents (7, 8, 10, 12). Sulfonamides have generally been considered the drugs of choice for the therapy of nocardiosis. However, we recently encountered two patients with pulmonary nocardiosis who required other therapy. In one of these patients the sulfonamides failed to influence the infection because of resistance of the organism, and in the other they could not be used because they produced serious drug reactions. Therapy selected on the basis of the in vitro susceptibility of the isolates of *N. asteroides* cultured from these patients resulted in clinical improvement and eradication of the organisms from the sputum and lungs. This experience prompted us to test the susceptibility of a number of strains to most of the available and useful antibiotics, including several new analogues which are under investigation but not yet approved for therapeutic use.

MATERIALS AND METHODS

Organisms. Ruth E. Gordon (Institute of Microbiology, Rutgers University, New Brunswick, N.J.) kindly provided transplants of 70 strains of *N. asteroides* on agar slants, and 5 strains were recently isolated, four of them by A. Kathleen Daly from patients with systemic nocardiosis at Boston City Hospital and one at another hospital. All were characterized as *N. asteroides* by the following criteria. They were gram-positive with branching filaments and were weakly acid fast when carbol fuchsin stain was decolorized with 1% sulfuric acid. No growth occurred in 0.4% gelatin. They did not hydrolyze casein and, with few exceptions, did not ferment lactose or mannitol (5).

Preparation of inoculum. Early in this study it became apparent that strains of *N. asteroides* differ markedly in their growth in liquid media. Some grew at 37 C to a uniformly turbid suspension of up to 10<sup>9</sup> colony-forming units (CFU) per ml within 48 hr, with the medium having a pH of 8.0 at that time, as described by Gordon and Hagan (6). In contrast, other strains tended to clump during growth or required up to 10 days to attain similar numbers of CFU. In this study, only cultures which achieved moderate density within 10 days were selected to test for susceptibility.

A loopful of the heavy growth from an agar slant of each organism was shaken into 2 ml of an enrichment broth prepared according to a formula suggested by R. E. Gordon (personal communication). This contained 5 g each of yeast extract, beef extract, glucose, and peptone per liter, and was adjusted to pH 7.0. The tubes were incubated at 37 C for 6 days. After thorough mixing in a Vortex-Genie mixer (Fisher Scientific Co.), 1.0 ml of the broth culture of *Nocardia* was transferred to a 125-ml Erlenmeyer flask con-
taining 10 ml of Mueller-Hinton broth (Difco) and some sterile glass beads. The flask was placed on a mechanical shaker and incubated for 4 days at 37 C. The resulting culture, with the beads, was transferred to sterile tubes and again mixed in the Vortex-Genie mixer. Homogeneous suspensions were thus generally obtained.

**Susceptibility tests.** Cultures that showed heavy growth and were very turbid were diluted 1:100 in Mueller-Hinton broth; slightly turbid cultures were diluted 1:10, and those that were still essentially clear were used undiluted in the wells of the inocula replicating apparatus (9). Four plates of serially diluted cultures were made at the time to obtain colony counts of the culture, and only the results of tests with those that yielded 10⁴ to 10⁶ CFU per ml were included in the data reported here. The replicator delivers 0.002 ml per inoculum, or 2 x 10⁴ to 1 x 10⁶ CFU of these cultures, on a series of Mueller-Hinton agar plates containing twofold dilutions of the antibiotics. The plates were examined after incubation for 48 hr at 37 C. The minimal inhibitory concentration (MIC) was considered to be the lowest concentration of antibiotic on which there was no growth visible with the aid of a hand lens (three times magnification). Antibiotic-free plates all showed heavy (confluent) growth before and after each series of tests.

**Antimicrobial agents.** The 45 antibiotics and chemicals used in the susceptibility tests, and their suppliers, included: 10 penicillins (benzylpenicillin, [Nutritional Biochemicals Corp.]; ampicillin, carbenicillin, amoxicillin [BRL-2235]; cloxacillin, and BRL-2288 [Beecham Laboratories]; oxacillin [Bristol Laboratories]; meticillin and epicillin [E. R. Squibb & Sons]; and nafcillin [Wyeth Laboratories]); 8 cephalosporins (cephalothin, cephalone, cephalaxin, and cephradine [Eli Lilly & Co.]; cepahirin [BLP-1322, Bristol Laboratories]; cepacetril [CIBA-Geigy]; cefazolin [Smith, Kline & French]; and cefoxitin [Merck, Sharp & Dohme]); 6 aminoglycosides (streptomycin [Eli Lilly & Co.]; neomycin [The Upjohn Co.]; kanamycin [Bristol Laboratories]; gentamicin [Schering Corp.]; and 2 new ones—tobra- mycin [Eli Lilly & Co.] and BB-K8 [Bristol Laboratories]; 7 tetracycline analogues (tetracycline, chlortetracycline, demeclocycline, and minocycline [Ledel Laboratories]; and oxtetracycline, meth- acycline, and doxycycline [Pfizer Co., Inc.]); 5 lincomycins (lincomycin, clindamycin, demethyl-clinda- mycin, depropyl-clindamycin, and U39,745E [The Upjohn Co.]); and 9 others of various classes (chloramphenicol [Parke, Davis & Co.]; erythromycin and vancomycin [Eli Lilly & Co.]; spectinomycin [The Upjohn Co.]; rifampin [CIBA]; sulfadiazine [Ledel Laboratories]; sulfamethoxazole [Hoffmann-La Roche, Inc.]; trimethoprim [Burroughs Wellcome]; and the combination of 1 part trimethoprim and 20 parts sulfamethoxazole).

These agents were supplied as powders or crystals, and solutions were freshly prepared at the time of the tests.

**Results**

The results of all of the tests for suscepti-

Plate density are displayed in Fig. 1-5. Related antibiotics have been grouped together in the same figure for the most part, but in some instances it was necessary, for clarity and convenience, to combine some unrelated agents.

**Penicillins and cephalosporins.** None of the 10 penicillins or the 8 cephalosporins was highly active against the strains of *N. asteroides* that were tested (Fig. 1 and 2). With few exceptions, the MIC was 12.5 µg/ml or greater. The most active of the penicillin-susceptible penicillins were amoxicillin, ampicillin, and cepillin, in decreasing order, as shown in the lower panel of Fig. 1; at a concentration of 50 µg/ml, they inhibited 74, 58, and 45% of the strains, respectively. Only about one-half of the strains were inhibited by 200 µg of benzylpenicillin/ml, and only a much smaller proportion were inhibited by that concentration of carbenicillin or BRL-2288. The penicillin-resistant penicillins were of similarly low activity (Fig. 2); a concentration of oxacillin, cloxacillin, nafcillin, and methicillin of 200 µg/ml inhibited 65, 37, 18, and 4% of the strains, respectively, and very few were inhibited by smaller concentrations.

Cephaloridine at a concentration of 50 µg/ml inhibited 59% of the strains (Fig. 2), and was the most active of the cephalosporins against most of the strains. Cefoxitin inhibited 40% at 50 µg/ml. Three of the new analogues—cephapirin, cephanone, and cefazolin—were nearly as active; all strains were inhibited by 100 µg of cepapirin/ml and by 200 µg of the other two/ml, as shown in upper panel of Fig. 1.

**Aminoglycosides.** The six aminoglycoside antibiotics differed widely in their relative activity against the strains tested (Fig. 3). In addition, the individual strains likewise varied widely in their susceptibility to each of these six antibiotics. A new member of this class, BB-K8 (Bristol Laboratories), was the most active; it inhibited all strains at a concentration of 25 µg/ml and 50% of them at 63 µg/ml. The other new aminoglycoside, tobramycin, inhibited one-half of the strains at 12.5 µg/ml, but one-fourth of them were resistant to 50 µg/ml. Of the four aminoglycosides that are in current use, gentamicin was the most active, one-half of the strains being susceptible to 12.5 µg/ml and 75% being inhibited by 50 µg/ml.

**Lincomycin analogues.** As shown in Fig. 4, lincomycin, clindamycin, and demethyl-clinda- mycin were, for the most part, inactive; only occasional strains were inhibited by 100 µg/ml, and one-half to three-fourths of the strains were resistant to 200 µg/ml. Depropyl-clindamycin and U39,745E were somewhat more active, 44% of strains being inhibited by 50 µg or less of the former/ml and 38% by 25 µg or less of the
Fig. 1. Susceptibility of Nocardia asteroides to six penicillinase-susceptible penicillins—penicillin G (benzylpenicillin), ampicillin, carbenicillin, and three newer semisynthetic analogues, namely, amoxicillin, epi-
cillin, and BRL-2288 (shown in upper panel), and to 6 cephalosporins—cephalothin, cephalaxin, and four
newer analogues, namely, cephapirin, cephanone, cefazolin, and cephaererile (shown in lower panel). Other
penicillins and cephalosporins are shown in Fig. 2.

Fig. 2. Susceptibility of 27 strains of Nocardia asteroides to four penicillinase-resistant penicillins—methi-
cillin, oxacillin, cloxacillin, and nafcillin; to two cephalosporins—cephaloridine and cefoxitin; and to chloram-
phenicol.
Fig. 3. Susceptibility of Nocardia asteroides to 12 antimicrobial agents: 4 aminoglycosides currently in general use—streptomycin, neomycin, kanamycin, and gentamicin; 2 new aminoglycosides—tobramycin and BB-K8 (Bristol Laboratories); and spectinomycin, rifampin, trimethoprim (TMP), sulfamethoxazole (SMZ), sulfadiazine, and a combination of TMP + SMZ in a ratio of 1:20. Maximal concentrations employed varied from 200 to 1,600 μg/ml, as indicated by the parallel vertical lines at the end of the curve representing each antibiotic.

Fig. 4. Susceptibility of strains of Nocardia asteroides to erythromycin, vancomycin, lincomycin, clindamycin and three new clindamycin derivatives, namely, depropyl-clindamycin, demethyl-clindamycin, and U-39, 745E.
latter/ml.

**Tetracyclines.** The most impressive finding was the nearly uniform susceptibility of all strains of *N. asteroides* to each of the seven tetracycline analogues that were tested and the marked differences in the relative activity of the individual analogues (Fig. 5). Only methacycline showed a bimodal distribution of the MIC values, 55% of the strains being inhibited by 12.5 μg/ml, and the MIC for most of the others was 100 μg/ml. By far the most active, and quite uniformly so, was minocycline; the MIC for 90% of the strains was 1.6 or 3.1 μg/ml, and all were susceptible to 6.3 μg/ml. Doxycycline ranked next in activity, but against all of the strains the MIC was four to eight times that of minocycline. Demeclocycline was the least active; the MIC for 50% of strains was 25 to 100 μg/ml, and almost all of the rest were inhibited by 200 μg/ml. Chlortetracycline was nearly as active as doxycycline, whereas the activity of oxytetracycline and tetracycline approached that of demeclocycline. Increasing the size of the inoculum had very little effect on the MIC of minocycline.

**Other antimicrobial agents.** Erythromycin (Fig. 4) was highly active against 42% of the strains (MIC, 0.4 to 0.8 μg/ml); for another 12%, the MIC was 3.1 to 12.5 μg/ml, and the remaining 46% were resistant to 100 μg/ml. Rifampin (Fig. 3) was quite active against about one-fifth of the strains (MIC, 6.3 μg or less/ml), but the MIC for the others was 100 to 400 μg/ml. Spectinomycin (Fig. 3) showed a trimodal distribution: the MIC was 6.3 to 25 μg/ml for one-fourth of the strains and 100 to 200 μg/ml for one-half of the strains; the other one-fourth of the strains were resistant to 800 μg/ml. Chloramphenicol (Fig. 2) varied widely in its activity: the MIC for three-fourths of the strains ranged from 12.5 to 100 μg/ml, and the other strains were resistant to the latter concentration. Vancomycin (Fig. 4) inhibited 15% of the strains at 0.8 μg/ml, but the MIC of the rest ranged from 12.5 to 100 μg/ml.

With the inoculum and medium used in our tests, we could not confirm the in vitro susceptibility of the strains of *N. asteroides* to sulfonamides (4). Sulfadiazine and sulfamethoxazole failed to inhibit all but a few of the strains in concentrations up to 2,000 μg/ml (200 mg/100 ml). However, when the inoculum was decreased to 10 to 100 CFU, many strains were susceptible to 6.3 to 50 μg/ml. Fewer than 20%

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**Fig. 5.** Susceptibility of 35 strains of *Nocardia asteroides* to seven tetracycline antibiotics.
of strains were inhibited by trimethoprim in concentrations up to 100 \(\mu g/ml\), and the combination of trimethoprim with 20 times as much sulfamethoxazole decreased the MIC of trimethoprim for some strains only two- to fourfold, confirming the observations reported by Black and McNellis (3).

**Studies of synergy.** The combination of trimethoprim with sulfamethoxazole mentioned above was tested only in a single ratio. A checkerboard arrangement of serial dilutions of each of two antimicrobial agents in a replica inoculator was used to test strains of *N. asteroides* with two other drug combinations. Minocycline plus sulfadiazine failed to show any potentiality against any of the strains, and, indeed, showed some suggestion of antagonism against some of them. On the other hand, the combination of erythromycin plus ampicillin showed marked synergy against nearly all strains tested. The results of the tests with this combination are reported in detail separately.

**DISCUSSION**

Several aspects of the studies presented deserve comment. The difficulties in assuring uniform dispersal of cultures of *Nocardia* have been noted by others (4). The differences in the numbers of strains tested with the individual antibiotics were largely a matter of convenience, but were also related to the amount of growth attained at the time of the tests. Nevertheless, it is clear from the results presented that there were wide variations among strains in their susceptibility to nearly all of the antibiotics, and that the different antibiotics, including those that are closely related in chemical structure, also varied markedly in their activity against the individual strains, despite their being tested at the same time and under the same controlled conditions. It is obvious, therefore, that if the in vitro activity of antimicrobial agents is a major criterion for the choice of therapy, it is essential to test the infected patient’s organism for susceptibility, particularly when the patient is not showing a favorable bacteriological and clinical response to the original agent selected, usually a sulfonamide, or when that agent cannot be used because it produces drug reactions. Table 1 lists the range and median of the MIC of each antibiotic and the individual MIC for three strains from our patients.

The ultimate criterion for the proper choice of therapy is the effect it produces in the infected patient. We are presenting elsewhere observations made on two patients with pulmonary nocardiosis in whom sulfonamides could not be used effectively—in one because they failed to produce any favorable bacteriological or clinical effect and in the other because they produced severe drug reactions. In each of these patients, elimination of the *Nocardia* strain from the sputum and lungs, as well as marked regression in the pulmonary lesions, followed therapy selected on the basis of susceptibility tests. This was accomplished with the combination of erythromycin and ampicillin in the first patient, and with minocycline in the other. We were unable to explain the basis for the “continuous variations” in MIC of the strains tested with some antibiotics, for example, ampicillin, amoxicillin, and epichillin (Fig. 1, lower panel), in contrast to the bimodal distribution of the MIC of others, such as spectinomycin, rifampin, and neomycin (Fig. 3), erythromycin (Fig. 4), or methacycline (Fig. 5), as compared with the more uniform susceptibility of all strains to minocycline and most of the other tetracyclines (Fig. 5).

Tests for susceptibility of *Nocardia* were also carried out with standard medicated discs by the Bauer-Kirby method (2) modified by the use of cultures standardized by actual counts of the number of CFU. These disc tests were applied to minocycline, tetracycline, ampicillin, and erythromycin, and in each instance they showed a good correlation with the results of the agar dilution method, indicating the feasibility of using the disc susceptibility tests for the choice of antibiotics against nocardial infections. However, as previously reported from this laboratory for strains of *Staphylococcus aureus* (1), it was shown that the tetracycline disc could not be used to identify strains susceptible to minocycline. Likewise, the minocycline disc did not distinguish tetracycline-resistant from tetracycline-susceptible strains. Also, as in the agar dilution method in which the replica inoculator was used, 23.75-\(\mu g\) discs of sulfamethoxazole showed no clear zones, confirming the resistance of the strains demonstrated by the dilution method. With 300-\(\mu g\) sulfasoxazole discs only 2 of 24 strains showed any clear zone.

M. L. Littman and L. E. Feingold (Abstr., 11th Intersci. Conf. on Antimicrob. Ag. Chemother., p. 59) reported that 70 to 82% of strains of *Nocardia* (several species) were susceptible to each of six tetracyclines by a disc diffusion test with an agar overlay, but they did not include minocycline nor any quantitative comparison. M. G. Orfanakis, H. Wilcox, and C. B. Smith (Antimicrob. Ag. Chemother. 1:215–220)
reported synergistic action of ampicillin and trimethoprim against two of four isolates of N. asteroides and clinical improvement with this combination in four immunologically suppressed patients who had pulmonary infections.

It was not our intention to be complete and include all available antibiotics. We limited this study primarily to the common classes of antibiotics other than the polypeptides and to the antimycobacterial agents. In particular, we did not test fusidic acid (3, 4) and capreomycin (11), which have been found active in vitro against some strains of Nocardia.

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LITERATURE CITED


