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Factors Influencing Susceptibility of *Nocardia* Species to Trimethoprim-Sulfamethoxazole

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Demonstration of synergism between trimethoprim and sulfamethoxazole against 10 *Nocardia* isolates was found to be critically dependent upon the isolate, the duration of incubation, and the trimethoprim-sulfamethoxazole ratio. Inoculum effect was not significant. The trimethoprim-sulfamethoxazole ratio in the commercial, fixed-dose combination was found to contain too little trimethoprim to be optimal for *Nocardia*.

Failure of many cases of nocardiosis to respond to therapy has prompted a continued search for more effective chemotherapeutic agents. Because sulfadiazine and its congeners have been the most effective drugs, introduction of the combination of sulfamethoxazole (SMZ) and trimethoprim (TMP) soon led to its evaluation in nocardiosis. Anecdotal accounts of success (1, 2, 8-12) have been encouraging, but these reports failed to prove whether or not TMP contributed to the successful result. In vitro studies have been conflicting, with four studies finding synergism between SMZ and TMP (3, 5, 9, 11) while another did not (4). All the studies have agreed that the minimal inhibitory concentration (MIC) of TMP alone was high, usually 20 to 50 μg/ml, compared to the obtainable blood levels of the drug. For example, peak TMP blood levels following administration of 160 mg were 1.5 μg/ml (13). The reason for these discrepant results between laboratories has been unclear. The current study evaluates methodological factors influencing assessment of TMP-SMZ synergism in *Nocardia* and offers an explanation of the previous discrepant results.

MATERIALS AND METHODS

Isolates. Source and identity of the *Nocardia* isolates is given in Table 1. Species identification was achieved by colony morphology, partial acid fastness, casein hydrolysis, and assimilation of tyrosine, xanthine, and hypoxanthine.

Identity of isolates 3 and 10 was confirmed by Ruth Gordon, Rutgers University, and that of isolate 9 by the Center for Disease Control, Atlanta, Ga.

Inocula. Isolates were maintained by weekly subculture on blood-yeast extract (Difco Laboratories, Detroit, Mich.). Prior to use, the isolates were subcultured into Middlebrook 7H9 broth and incubated at 37°C for 4 to 5 days. Growth was ground in a sterile mortar and pestle and briefly gravity sedimented, and the finely dispersed supernatant was recovered. The concentration of microorganisms was adjusted spectrophotometrically, and the number of viable particles was enumerated by pour plates, using Mueller-Hinton agar (Difco).

Drugs. TMP (lot 64789) and SMZ (lot 58742) were kindly provided by Burroughs Wellcome, Research Triangle Park, N.C. Stock solutions of TMP were prepared as 1,000 μg/ml in 0.05 N lactic acid and stored at −13°C. SMZ powder was dissolved in a small quantity of aqueous 10% NaOH, diluted to 500 μg/ml, and stored at −13°C. Final concentrations of SMZ in agar ranged from 0.19 to 25 μg/ml when used alone and from 0.098 to 12.5 μg/ml in all combinations. TMP concentrations were 0.78 to 100 μg/ml when used alone and 0.0049 to 0.625, 0.039 to 5.0, and 0.31 to 40 μg/ml in the three combinations. Both drugs were employed in twofold dilution steps within the range studied. In every test, concentrations chosen bracketed the MIC.

Susceptibility testing. One or both drugs were dissolved in a single lot of Mueller-Hinton agar, and the agar surface was inoculated with two dilutions of inoculum in 0.025 ml. Sizes of these inocula are given in Table 1. Plates were incubated in plastic bags at 37°C and observed for the presence or absence of growth at 2 and 7 days. Suitability of this lot of Mueller-Hinton agar for SMZ and TMP susceptibility testing was determined by comparing the results in isolates 5 and 9, using Mueller-Hinton agar both with and without the addition of 7.5% lysed horse blood. Growth of one isolate was slightly less in the blood-containing agar, and small amounts of growth were more difficult to see in blood-containing agar because of the darker color. Growth inhibition by TMP, SMZ, and three combinations of TMP and SMZ was not influenced by the addition of horse blood.

RESULTS

Results of testing SMZ and TMP alone, using two different inocula and both 2 and 7 days of

incubation, are shown in Fig. 1 and 2. With the low inoculum and 2-day incubation, all isolates were susceptible to SMZ at concentrations less than or equal to 6.25 μg/ml. Under those same conditions, only 1 of 10 isolates was susceptible to concentrations of TMP readily obtainable in serum, i.e., 1.5 μg/ml. With both TMP and SMZ, high inocula and prolonged incubation decreased the magnitude of drug effect.

TABLE 1. *Inocula for susceptibility testing*

Isolate no.	Species	Site obtained	Inocula (CFU)[a]
1	*N. asteroides*	Sputum	25
2	*N. asteroides*	Finger	26
3	*N. asteroides*	Sputum	5
4	*N. asteroides*	Urine	52
5	*N. asteroides*	Sputum	170
6	*N. asteroides*	Bronchial wash	49
7	*N. asteroides*	Sputum	156
8	*N. asteroides*	Maxillary sinus	131
9	*N. asteroides*	Sputum	8
10	*N. caviae*	Elbow	6

[a] Colony-forming units (CFU) for the low inocula are listed. High inocula were 1,000× these numbers.

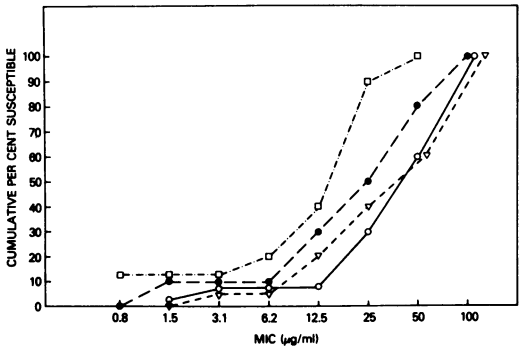

FIG. 1. *TMP susceptibility of 10 Nocardia isolates. Symbols: (□) low inocula, 2 days; (▽) high inocula, 2 days; (●) low inocula, 7 days; (○) high inocula, 7 days.*

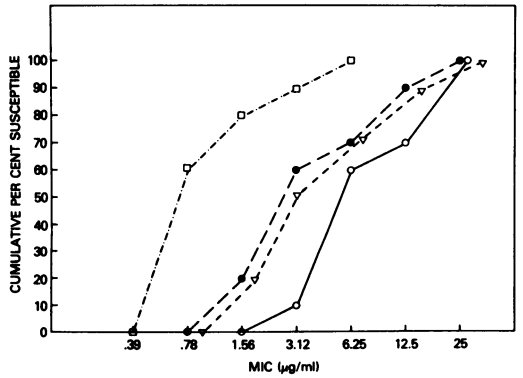

FIG. 2. *SMZ susceptibility of 10 Nocardia isolates. Symbols: (□) low inocula, 2 days; (▽) high inocula, 2 days; (●) low inocula, 7 days; (○) high inocula, 7 days.*

Three different mixtures of TMP and SMZ were also studied. The TMP-SMZ ratio of 1:20 often has been used in susceptibility testing because this approximates the ratio of the serum drug concentrations when the commercially available fixed-dose combination is administered. This weight ratio of TMP-SMZ and two other mixtures, containing 8 and 64 times as much TMP, were tested for their MIC. The MIC was used to determine the fractional inhibitory concentration (FIC) (6) of each component of the mixture. Then the sum of the two FICs was determined, a number which Elion et al. have termed the "FIC index" (6). Results are given in Tables 2 and 3. An FIC index of 1.0 indicates an additive effect, whereas a higher index designates antagonism and a lower index, synergism. For example, if inhibition was achieved when each component was used at a concentration equal to 1/4 MIC of the component tested alone, the FIC index would be 0.5. This will be termed here "definite synergy." Conversely, were each drug needed at a full MIC in the mixture, the FIC index would be 2.0. This will be termed "definite antagonism." The results show that two factors favored demonstration of synergy: increased TMP in the mixture and prolonged incubation. Modification of TMP concentration had the greatest effect. At the highest TMP-SMZ ratio, definite synergism could be shown with eight or nine of the isolates, using three of the four test conditions. With the lowest TMP-SMZ ratio, definite synergism was seen in only two to five isolates with any of the four conditions, and one to two isolates showed definite antagonism.

A four-way analysis of variance was done (through the kind assistance of David Alling) to ascertain the relative contribution of the various factors in the experimental design to the variability observed in the FIC index. Inoculum effect was not significant ($F < 1$; df 1, 106; $P > 0.25$). As might be expected, similar analysis showed the major sources of variability to be the TMP-SMZ ratio, duration of incubation, and isolate. Details of the analysis are provided in Table 4.

DISCUSSION

The results in Tables 2 and 3 indicate that synergism between TMP and SMZ could best be demonstrated against *Nocardia* species with prolonged incubation, a condition which decreased antimicrobial effects of either drug when

TABLE 2. *FIC indexes of Nocardia species using mixtures with different TMP-SMZ ratios*

Isolate	2 days incubation						7 days incubation					
	High inoculum			Low inoculum			High inoculum			Low inoculum		
	3.2:1	1:2.5	1:20	3.2:1	1:2.5	1:20	3.2:1	1:2.5	1:20	3.2:1	1:2.5	1:20
1	0.30	0.51	0.25	0.35	0.35	0.13	0.35	0.53	0.51	0.30	0.51	0.99
2	0.45	0.55	1.00	0.55	0.20	0.05	0.15	0.27	1.00	0.30	1.00	0.50
3	0.75	2.98	5.90	1.12	1.12	1.12	0.70	1.04	1.99	1.10	2.02	4.00
4	0.16	0.16	0.51	0.60	1.02	2.00	0.16	0.30	0.51	0.06	0.13	0.12
5	0.17	0.26	0.51	0.08	0.14	0.11	0.22	0.27	0.51	0.26	0.17	0.26
6	0.29	0.51	0.51	0.55	1.01	1.01	0.29	0.51	1.00	0.26	0.49	0.99
7	0.60	2.50	4.98	1.48	3.00	6.00	0.22	0.11	0.26	0.14	0.28	0.50
8	0.11	0.26	0.49	0.34	0.51	0.99	0.08	0.07	0.25	0.22	0.26	0.49
9	0.34	0.70	1.40	0.54	0.54	0.54	0.32	0.55	0.50	0.34	0.52	1.01
10	0.35	0.52	1.11	1.03	0.99	1.05	0.17	1.03	2.13	0.17	0.50	0.50
Mean index	0.35	0.90	1.67	0.66	0.89	1.30	0.27	0.47	0.86	0.31	0.59	0.94
Group mean index		0.97			0.95			0.53			0.61	

TABLE 3. *FIC indexes of Nocardia species isolates*

FIC Index	No. of isolates in each FIC index category[a]											
	2 days incubation						7 days incubation					
	High inoculum			Low inoculum			High inoculum			Low inoculum		
	3.2:1	1:2.5	1:20	3.2:1	1:2.5	1:20	3.2:1	1:2.5	1:20	3.2:1	1:2.5	1:20
≥2.00	0	2	2	0	1	2	0	0	1	0	1	1
1.0–1.99	0	0	3	3	3	3	0	2	3	1	1	1
0.51–0.99	2	5	3	4	3	2	1	3	3	0	2	3
≤0.5	8	3	2	3	3	3	9	5	3	9	6	5

[a] Out of 10 isolates tested, based on data in Table 2.

TABLE 4. *Analysis of variance*

Source of variation	df	Sum of squares	Mean square
Among ratios	2	12.77	6.38[a]
Among isolates	9	36.98	4.11[b]
Between incubation times	1	4.51	4.51[c]
Between inoculum levels	1	0.03	0.03
Error	106	67.16	0.634
Total	119	121.45	

[a] $F = 10.1$; $P < 0.001$.
[b] $F = 6.48$; $P < 0.001$.
[c] $F = 7.11$; $P < 0.01$.

used alone (Fig. 1 and 2). The results in Tables 2 and 3 also suggest that the relative nonsusceptibility of *Nocardia* species to TMP, compared with bacteria, made the use of the usual TMP-SMZ ratio of 1:20 inappropriate for *Nocardia*. This phenomenon was studied by Bushby in 21 isolates of *Nocardia* (5). Although the species, inoculum, and incubation time were not noted, the results well demonstrated the effect of the TMP-SMZ ratio on synergy. The FIC index was ≤0.5 in all 21 isolates with a TMP-SMZ ratio of 9:1. The FIC index was that low in only 7 of 21 isolates at a TMP-SMZ ratio of 1:9. The most discouraging report concerning synergy between TMP and SMZ against *Nocardia* (4) employed TMP-SMZ ratios no higher than 1:1. In that report, the FIC index was 0.5 or less in only one of 12 *Nocardia* species, using a 1:1 ratio, an inoculum of 250 to 2,500 colony-forming units, and 2 days of incubation. This is not far different from the results reported here with a TMP-SMZ ratio of 1:2.5, inocula of 5 to 170 colony-forming units, and 2 days of incubation, in that 3 of 10 isolates had an FIC index of ≤0.5. The results of Wallace et al. with 37 *Nocardia* isolates also support such results (14). These investigators used agar dilution, an inoculum of 100 colony-forming units, Mueller-Hinton agar, and 2 days of incubation. Although TMP alone was not tested, the MIC of TMP-SMZ at a ratio of 1:20 was more than twofold less than SMZ alone in less than 10% of strains.

Of all the factors influencing the demonstration of synergy against *Nocardia* species, therapeutic relevance is most obvious with the TMP-SMZ ratio. The commercial mixture ap-

pears to contain too little TMP to contribute substantially to SMZ effect. Taking the 2-day, low-inoculum result, antagonism was as likely as synergism. There is nothing in the reported cases, reviewed elsewhere (7), to dispel this notion.

The in vitro results suggest the desirability of using at least as much TMP as SMZ in therapy of nocardiosis, but the toxicity of such mixtures is unexplored. The usual treatment period of nocardiosis is 6 to 12 months. When the TMP-Sulfa combination has been given to adults for prolonged periods with TMP doses of 0.5 to 1.0 g, reversible hematological changes have occurred (15) It remains to be seen whether adequate TMP doses can be given to exploit the synergism demonstrated in vitro.

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