Sensitivity of *Nocardia* to trimethoprim and sulphonamides *in vitro*

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SYNOPSIS Studies *in vitro* of the sensitivity to trimethoprim and sulphonamides of nine strains of *N. asteroides*, two strains of *N. caviae*, and one of *N. blackwellii* are presented. No unequivocal evidence of synergism was found. Despite this, the inclusion of trimethoprim in the drug regime when sulphonamides are used in the treatment of nocardiosis is suggested on empirical grounds.

In recent years it has been demonstrated that trimethoprim (2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine) reacts with sulphonamides in a manner which is strongly synergistic and that drugs, which are themselves bacteriostatic, achieve a bactericidal effect when used together. The pharmacology of this drug combination has been thoroughly evaluated *in vitro* and in laboratory animals by Bushby and Hitchings (1968). Darrell, Garrod, and Waterworth (1968) confirmed the *in-vitro* findings of the above authors and reported favourably on the use of a sulphonamide-trimethoprim regime in successfully treating respiratory infections, urinary tract infection, and Gram-negative septicaemia. More recently, there have been further reports of success in the treatment of urinary tract infection (Grüneberg and Kolbe, 1969; Reeves, Faiers, Pursell, and Brumfitt, 1969), of neonatal meningitis due to *Escherichia coli* (Morzaria, Walton, and Pickering, 1969), and it has been found (Akinkugbe, Lewis, Montefiore, and Okubadejo, 1968) that a combination of sulphamethoxazole and trimethoprim was equal in value to chloramphenicol in the treatment of typhoid fever.

There have, however, been no reports to date on the use of a sulphonamide-trimethoprim mixture in the treatment of *Nocardia* infections where, despite the advent of many potent new antibiotics, sulphadiazine remains the drug of choice (Strauss, Kligman, and Pillsbury,

1951; Neu, Silva, Hazen, and Rosenheim, 1967; Shuster, Klein, Pribor, and Kozub, 1967). Peabody and Seabury (1960), however, in an extensive review of the therapy of actinomycosis and nocardiosis have suggested that better results might be obtained if sulphadiazine were to be used along with another chemotherapeutic drug in the treatment of the latter condition. Trimethoprim would appear on theoretical grounds to be the obvious choice of drug for this purpose, and the following paper, in an attempt to substantiate this theory, presents an account of studies *in vitro* of the effect of sulphonamide-trimethoprim mixtures on nine strains of *Nocardia asteroides*, two of *N. caviae*, and one of *N. blackwellii*.

Materials and Methods

The strain N1 was isolated in this laboratory from a fatal case of nocardiosis, and the strain NHS was isolated by Dr H. Singh from a fatal case at another hospital in this area. The origin of the other species of *Nocardia* is given in Table I. *Staphylococcus aureus* (CN 491) and *Escherichia coli* (CN 314) were included as controls.

The trimethoprim, sulphamethoxazole, and sulphadiazine were freshly prepared before each experiment.

Trimethoprim, available as trimethoprim lac-

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Strain	Species	Source
N1	<i>N. asteroides</i>	Fatal case of nocardiosis
NHS	<i>N. asteroides</i>	Fatal case of nocardiosis
N8595	<i>N. asteroides</i>	National Collection of Type Cultures
N6761	<i>N. asteroides</i>	National Collection of Type Cultures
N770	<i>N. asteroides</i>	Mycology Reference Laboratory
CN2470	<i>N. asteroides</i>	Wellcome Bacterial Collection
RG874	<i>N. asteroides</i>	Institute of Microbiology, New Jersey
RG886	<i>N. asteroides</i>	Institute of Microbiology, New Jersey
W4	<i>N. asteroides</i>	Westminster Medical School
N1934	<i>N. caviae</i>	National Collection of Type Cultures
W2	<i>N. caviae</i>	Westminster Medical School
N630	<i>N. blackwellii</i>	National Collection of Type Cultures

Table I *Origin of Nocardia species*

Strain	Minimum Inhibitory Concentration Sulphamethoxazole + Trimethoprim				Trimethoprim	Sulpha- methoxazole
	Ratio					
	20:1	10:1	5:1	1:1		
N1	1:25	1:25	2:5	2:5	50	1:25
NHS	1:25	1:25	1:25	2:5	50	1:25
N8595	1:25	1:25	1:25	1:25	10	5
N6761	2:5	2:5	2:5	2:5	50	2:5
N770	2:5	2:5	2:5	2:5	50	2:5
CN2470	2:5	1:25	1:25	1:25	50	2:5
RG874	1:25	1:25	1:25	0:625	25	0:625
RG886	2:5	2:5	2:5	2:5	50	2:5
W4	2:5	1:25	1:25	1:25	25	1:25
N1934	2:5	1:25	1:25	1:25	50	2:5
W2	2:5	2:5	2:5	2:5	50	2:5
N630	1:25	1:25	1:25	1:25	25	1:25
<i>Staph. aureus</i>						
CN491	0:625	<0:312	<0:312	<0:312	<0:312	5
<i>Esch. coli</i>						
CN314	0:625	0:625	<0:312	<0:312	<0:312	10

Table II *Minimum inhibitory concentrations of sulphamethoxazole, trimethoprim, and various combinations*

S:T Ratio	MIC ($\mu\text{g/ml}$)		
	2:5 S:T	1:25 S:T	0:625 S:T
20:1	2:38:0:12	1:19:0:06	0:595:0:03
10:1	2:27:0:23	1:14:0:11	0:568:0:051
5:1	2:09:0:41	1:04:0:21	0:521:0:104
1:1	1:25:1:25	0:625:0:625	0:312:0:312

Table III *Amounts of sulphamethoxazole (S) and trimethoprim (T) in the combined MIC at the various ratios*

tate, was freely soluble in water. The sulphonamides were first dissolved in water at an alkaline pH by the addition of N/1 NaOH after which it was found possible to readjust the pH to 7.5 with N/1 HCl without precipitation occurring. From basic dilutions in nutrient broth (Oxoid-CM1) containing 10,000 $\mu\text{g/ml}$, tube dilutions

containing 2,000-500 $\mu\text{g/ml}$ and 200-6.25 $\mu\text{g/ml}$ were prepared for each drug. Aliquots of the 2,000 $\mu\text{g/ml}$ solutions of the three drugs were then taken and both the sulphamethoxazole and the sulphadiazine mixed with trimethoprim to give sulphonamide:trimethoprim ratios of 20:1, 10:1, 5:1, and 1:1, and, from each of these 2,000 $\mu\text{g/ml}$ mixtures, dilutions ranging from 2,000 to 6.25 $\mu\text{g/ml}$ were made as before.

Universals containing measured 18 ml aliquots of molten diagnostic sensitivity test agar (Oxoid-CM261) were allowed to cool to about 50°C to 60°C and, before pouring, 1 ml of lysed horse blood and 1 ml of one of the antibiotic dilutions were added to each giving a final antibiotic concentration ranging from 100 to 0.312 $\mu\text{g/ml}$.

A measured loopful (0.0025 ml) of a six-hour mechanically agitated culture in nutrient broth of each *Nocardia* strain was then inoculated on to every plate and the inoculum spread over an area of about 1 sq cm. Tween 80 was added to the broth in later experiments to reduce granularity of the broth culture and make it easier to standardize the dose of organisms, which were usually present in a concentration of 10^5 to $10^6/\text{ml}$. This procedure did not affect the interpretation of the results. Using the above dose, a heavy but not confluent growth was obtained and the minimum inhibitory concentration (MIC) was interpreted as in previous studies, namely, the lowest concentration of antimicrobial that inhibited at least 90% of the control growth (Bushby and Hitchings, 1968).

Results

The results, which were read after 48 hours incubation at 37°C, are detailed in Tables II and III.

From these it can be seen that the sulphonamide MICs are satisfactory and could be quite easily achieved *in vivo* by standard dosages. Raich, Casey, and Hall (1961), for example, showed that a blood sulphonamide level of 140 $\mu\text{g/ml}$ could be maintained by a dosage of 6 to 8 g of sulphadiazine per day, and Garrod and O'Grady (1968) report a peak plasma level of 100 $\mu\text{g/ml}$ after an initial dose of 4 g of sulphadiazine followed by 1 g four hourly. Stokes (1968) maintains that for most acute infections the average blood antibiotic level should exceed the MIC by a safety factor of 2 or 4, but for actinomycosis (and presumably nocardiosis) the safety factor should be higher. These criteria are satisfied by the present figures which show an average MIC of about 2.5 $\mu\text{g/ml}$ with a potential blood level of 100 to 140 $\mu\text{g/ml}$ ie, 20 to 30 times the MIC.

The sensitivity to trimethoprim is disappointing. In other series the average MIC for most of the bacteria tested was of the order of 1 $\mu\text{g/ml}$

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or less (Bushby and Hitchings, 1968; Darrell *et al*, 1968) whereas with the *Nocardia* in our series the MIC of trimethoprim was 50 µg/ml in eight out of the 12 strains tested, 25 µg/ml in three strains, and 10 µg/ml in the remaining strain. As Bushby and Hitchings (1968) were able to obtain peak serum levels of only 2 to 3 µg/ml of trimethoprim one hour after giving an oral dose of 250 mg, it must be concluded that the use of trimethoprim alone would be of no value in the treatment of nocardiosis.

In the case of *Neisseria gonorrhoeae*, it has been shown (Bushby and Hitchings, 1968) that there was a high degree of potentiation of sulphadiazine by trimethoprim despite the fact that these organisms are relatively resistant to the latter drug. The results shown in Tables II and III, however, indicate that this synergistic effect was not demonstrated unequivocally in any of the four sulphonamide:trimethoprim ratios tested. There did, however, seem to be some enhancement of the sulphonamide when this was used with an equal amount of trimethoprim but in only one or two cases was there more than a two-fold difference between the sulphonamide acting alone and the sulphonamide combined with trimethoprim.

Sulphamethoxazole is the sulphonamide of choice for use with trimethoprim as these drugs are absorbed and excreted at the same rate. We chose to test sulphadiazine as well as sulphamethoxazole because the former has been used most widely in the treatment of nocardiosis (Peabody and Seabury, 1960). Tested individually, the MIC of sulphadiazine is the same, or two-fold higher, than that of sulphamethoxazole, a finding which tallies with that of Neipp (1964) in his survey of comparative activities of sulphonamides *in vitro*. Sulphadiazine displayed the same lack of marked synergistic activity with trimethoprim as did sulphamethoxazole.

Discussion

At the time of publication, sulphadiazine remains the drug of choice in the treatment of nocardiosis (Strauss *et al*, 1951; Neu *et al*, 1967; Shuster *et al*, 1967). There has, however, been a recent report of the successful treatment of a case of cerebral nocardiosis using a combination of sulphonamides and cycloserine (Hoeprich, Brandt, and Parker, 1968), and Vasarinsh (1968) reported the successful treatment of a case of primary cutaneous nocardiosis using a combination of sulphadiazine, erythromycin, and sulfone. The fact that no unequivocal synergism with trimethoprim was seen *in vitro* was disappointing but might have been expected from the relatively high MIC of the latter for, in their study of urinary tract infection, Reeves *et al* (1969) showed that the failure of treatment

in all species tested was associated with a relatively high resistance to trimethoprim.

On the other hand, Wright and Grimble (1969) found that the cure rate in gonorrhoea was only 30% when treated with sulphamethoxazole but that when this was combined with trimethoprim the cure rate rose to 80% even although the MIC of trimethoprim for the gonococcus has been shown to be as high in some cases as that which we found for *Nocardia* (Bushby and Hitchings, 1968). Again, Csonka and Knight (1967) have shown that gonorrhoea can be successfully treated with a sulphonamide-trimethoprim mixture even although trimethoprim itself is useless in treating this condition. It must be remembered, however, that a synergistic effect of sulphonamide and trimethoprim on the gonococcus has been demonstrated *in vitro* (Bushby and Hitchings, 1968) whereas we have failed to demonstrate this convincingly with *Nocardia*.

The question of therapy is not merely of academic interest as nocardiosis is a chronic, disabling condition which even if correctly diagnosed and treated is still attended by a high morbidity and mortality. Hoeprich *et al* (1968) in a review of 148 cases of nocardiosis report a fatality rate of about 87% in patients with cerebral involvement and 10% in patients with uncomplicated pulmonary disease who had received sulphonamide therapy. In dealing with a condition of this severity, the present findings cannot justify any reduction in what is accepted as a reasonable dose of sulphonamide, viz, 6-8 g/day, although the addition to this regimen of 1 g/day of trimethoprim (which is the maximum clinically acceptable dose) would appear worthy of trial, as our laboratory findings suggest that a 1:1 sulphonamide:trimethoprim ratio (and, to a lesser extent a 5:1 ratio) does give some minor degree of synergism. The choice of sulphonamide used in treating nocardiosis will depend on whether or not trimethoprim is to be added to the regime. If it is, then sulphamethoxazole would be preferable; if it is not, then sulphadiazine should be chosen because of its proven efficacy.

It is hoped that this report will stimulate further investigation of this topic in an effort to reduce the morbidity and mortality of nocardiosis to a more acceptable level.

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Addendum

Since the completion of this work it has been observed that our original strain N1 is sensitive *in vitro* to 0.625 µg/ml of fucidin. At present we are in the process of investigating the comparative MICs of fucidin and sulphonamides for *Nocardia*.