ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1974, p. 344-353

Vol. 5, No. 3
Printed in U.S.A.

Synergistic Action of Ampicillin and Erythromycin Against *Nocardia asteroides*: Effect of Time of Incubation

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Received for publication 6 December 1973

Ampicillin and erythromycin were shown to act synergistically in vitro against the majority of strains of *Nocardia asteroides* tested. With these strains, the minimal inhibiting concentration (MIC) of each drug when combined was reduced 4- to more than 512-fold as compared with the MIC of each antibiotic acting individually against the same strains. The combined action against other strains was at least additive, and occasionally indifferent, but antagonism was not observed. The duration of incubation greatly influenced the MIC of erythromycin but had less effect on the action of ampicillin. Synergistic action was still demonstrable, although less frequently, when the period of incubation was increased from 48 to 72 h.

We recently encountered pulmonary nocardiosis in a recipient of a renal transplant in whom treatment with trisulfapyrimidines had to be stopped because of severe drug reactions before the progress of the infection was arrested. In vitro tests with the *Nocardia asteroides* strain from that patient showed ampicillin and erythromycin to be the most active of the antimicrobials tested. Treatment with both of these antibiotics in moderate doses eliminated *Nocardia* for the first time from the patient's sputum, and the pulmonary lesion regressed. In another patient with similar symptoms, in whom treatment with various sulfonamides in large doses failed to control the infection, minocycline (not used in the tests with the strain from the first patient) was most active in vitro against the strain of *N. asteroides* isolated and proved similarly effective in the therapy of that patient. The clinical and laboratory findings in these two patients are reported in detail elsewhere (1).

The observations in these two cases prompted an extensive study of the in vitro activity of a large number of strains of *N. asteroides* to most of the antimicrobials currently available for therapy and to several which were still under investigation. Minocycline proved to be the most uniformly active of the 45 drugs tested; ampicillin and the chemically related amoxicillin were the most active of the penicillins and cephalosporins; and erythromycin in a concentration of 0.8 μg or less per ml inhibited 40% of strains, and 25 μg/ml inhibited 53% of the strains.

We report here the results of studies in which strains of *N. asteroides* were tested simultaneously for susceptibility to erythromycin and ampicillin, separately and in combination, by an agar-dilution method.

MATERIALS AND METHODS

The strains of *N. asteroides* were selected from the same collection of cultures that had been used previously (2), except that two additional clinical isolates were included. The methods were essentially the same as those used previously. Preparation of the cultures involved initial cultivation in an enrichment broth and selection of those strains which grew heavily within 6 days at 37 C; these were subcultured in Mueller-Hinton broth (Difco), and only strains that appeared to be heavily grown after 96 h of incubation were selected for sensitivity testing. These cultures generally contained about 10^8 to 10^9 colony-forming units (CFU) per ml, but some contained less than 10^8 CFU. All cultures in liquid media were incubated on a mechanical shaker, and the final cultures were further agitated on a Vortex Genie (Fisher Scientific) before being placed in the wells of a Steers inocula-replicator (11).

Plates of Mueller-Hinton agar were prepared with serial dilutions of each antibiotic alone and also with combinations of paired dilutions over the entire range of each antibiotic. Control cultures on antibiotic-free agar all showed moderate to heavy confluent growth. The minimal inhibiting concentration (MIC) was taken to be the concentration(s) of antibiotic(s) on which there was no growth visible with the aid of a hand lens (3×) after incubation for 48 ± 4 h in the

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first set of tests, after 72 h in the second, and after 24, 48, and 72 h (each ±4 h) in the third. When a combination produced inhibition with a low concentration of one antibiotic and a reduced but still high concentration of the other (as compared with the MIC of each antibiotic used alone), and the second antibiotic in a lower concentration acted similarly to the first, the results of both combinations are reported (as shown for tests 12, 33, 35, and 52 in Table 1).

RESULTS

Series of tests were carried out on three occasions, the second 11 weeks after the first, and the third 9 months later. On the first occasion, 28 strains satisfied the criteria of growth for inclusion, but 5 of the strains were inhibited by the lowest concentration of erythromycin used (0.4 μg/ml) and hence were unsatisfactory for tests of synergy with ampicillin. The results with these five strains are omitted, but they did not demonstrate any antagonism by ampicillin. Lower concentrations of erythromycin (to 0.1 μg/ml) were included in the second group of tests done with 35 strains, 18 of which had also been tested in the first group. Overall, therefore, 58 tests of susceptibility to ampicillin and erythromycin alone and in combination were performed with 40 strains in the first two experiments (Table 1). In 16 instances, two results are listed for the MIC of the combination; these essentially represent the two most favorable points on the isobolograms.

Synergy, defined as inhibition by one-fourth or less of the concentration of each of the two antibiotics when combined, as compared with the MIC of each antibiotic alone, was demonstrated in 30 of the 58 tests performed (52%). In 4 of these (nos. 12, 33, 35, and 52), the synergistic MIC of the combination is listed twice because each antibiotic inhibited in a considerably lower concentration when combined with the other, in addition to inhibiting growth with one-fourth or less of both drugs than anticipated from the MIC of the individual agents.

The combined action of ampicillin and erythromycin against three (5%) of the strains tested (nos. 3, 15, and 45) was indifferent in that the MIC of each was not influenced when combined with the MIC or lower concentration of the other.

In the other 25 tests (43%), the combined action was at least additive in that the combination was inhibitory when one-half of the MIC of one was combined with one-fourth or less of the MIC of the other. With strains which were not inhibited by the highest concentration of the antibiotics used in these two sets of tests (200 μg/ml), inhibition by 200 μg/ml in the combination was considered a twofold reduction and hence additive, even though one-fourth or less of the MIC of the other was required. However, the reduction may in fact have been greater and hence the combined action may have been synergistic. Some strains, for example (nos. 36, 37, and 40), which were not inhibited by 200 μg/ml concentrations of either of the two antibiotics alone, were inhibited by 200 μg of each per ml when combined with very low concentrations of the other; the same strains in the first set of tests (nos. 6, 7, and 8, respectively) were acted on synergistically by the combination.

Antagonism, or interference, was not observed in any instance.

A comparison and analysis of the MICs for organisms acted on synergistically by ampicillin and erythromycin and those on which the combined action was additive or indifferent is summarized in Table 2. The median and mean MICs of ampicillin alone for the former were about one-half of those for the latter, and for erythromycin alone they were one-sixteenth of the MICs for the strains not acted on synergistically. The reduction in the concentrations of both ampicillin and erythromycin, when they acted synergistically in combination, varied widely with the strains; the mean reduction of each was more than 16-fold.

Comparison of results of two sets of test data. Synergy was demonstrated with a considerably larger proportion of the 23 strains in the first series of tests (65%) than with the 35 strains in the second series (42%); addition, or possible synergy, was observed twice as often in the second series (54%) as in the first (26%). This is shown in the upper portion of Table 3.

In both the first and second sets of tests, the MIC of ampicillin alone for about half of the strains was 25 μg or less per ml. All but one of the remaining strains were inhibited by 50 or 100 μg of that antibiotic per ml in the first set; in the second set, about one-fourth of the strains were inhibited at 50 or 100 μg/ml and the rest grew well in 100 μg of ampicillin per ml. In the first set, erythromycin inhibited about half of the strains (12 of the 23) in concentrations of 25 μg or less per ml, but it did not inhibit the others even when a concentration of 100 μg/ml was used. In the second set of tests, the MIC of erythromycin for nearly one-third of the 35 strains was 25 μg or less per ml; of the remaining strains, most were not inhibited by 200 μg/ml, and two were inhibited by 50 or 100 μg/ml (Table 3, section A).

The results obtained with the 18 strains that

TABLE 1. *Susceptibility of Nocardia asteroides to ampicillin (A), erythromycin (E), and both (A + E)*

Test no.	MIC (μg/ml)			Fold decrease (A + E)	Test no.	MIC (μg/ml)			Fold decrease (A + E)
	A	E	A + E			A	E	A + E	
1	25	25	1.6 + 6.3	16 + 4	31[a]	12.5	0.4	0.4 + 0.1	32 + 4
2	25	25	6.3 + 6.3	4 + 4	32[a]	12.5	1.6	0.4 + 0.1	32 + 16
3	25	100	25 + 6.3	—[b]	33[a]	50	>200	0.4 + 25	128 + ≥8
4	100	50	25 + 6.3	4 + 8				1.6 + 0.1	32 + >2,000
5	100	25	25 + 6.3	4 + 4	34[a]	12.5	0.4	1.6 + 0.1	8 + 4
6	100	100	25 + 25	4 + 4	35	100	0.4	6.3 + 0.05	16 + 8
7	100	100	25 + 25	4 + 4				1.6 + 0.1	64 + 4
8	>200	100	6.3 + 25	≥32 + 4	36[6]	>200	>200	200 + 0.4	≥2 + ≥500[c]
9	25	3.1	6.3 + 0.4	4 + 8				0.4 + 200	≥500 + ≥2[c]
10	12.5	50	0.4 + 0.4	32 + 128	37[7]	>200	>200	200 + 0.4	≥2 + ≥500[c]
11	100	25	0.4 + 0.4	256 + 64				0.4 + 200	≥500 + ≥2[c]
12	25	25	1.6 + 1.6	16 + 16	38	12.5	0.4	0.4 + 0.1	32 + 4
			6.3 + 0.4	4 + 64	39	12.5	25	0.4 + 0.1	32 + 256
13	6.3	12.5	1.6 + 1.6	4 + 8	40[8]	>200	>200	200 + 25	≥2 + ≥8[c]
14	12.5	12.5	6.3 + 0.4	2 + 32[c]				0.4 + 200	≥500 + ≥2[c]
15	3.1	1.6	3.1 + 0.4	—[b]	41	6.3	6.3	0.4 + 0.4	16 + 64
16	100	50	25 + 6.3	4 + 8	42[10]	25	0.2	0.4 + 0.025	64 + 8
17	100	100	25 + 25	4 + 4	43[11]	25	0.4	0.4 + 0.1	64 + 4
18	50	100	25 + 1.6	2 + 64[c]	44[12]	25	>200	0.4 + 200	64 + ≥2[c]
19	12.5	12.5	6.3 + 0.4	2 + 32[c]	45[13]	25	200	—	—[b]
			1.6 + 6.3	8 + 2[c]	46[14]	50	>200	0.4 + 200	256 + ≥2[c]
20	50	50	6.3 + 25	8 + 2[c]	47	12.5	3.1	0.4 + 0.1	32 + 32
			25 + 0.4	2 + 128[c]	48[19]	25	>200	0.4 + 200	64 + ≥2[c]
21	50	0.8	6.3 + 0.4	8 + 2[c]	49[16]	200	>200	100 + 0.05	2 + ≥4,000[c]
22	50	50	25 + 6.3	2 + 8[c]				0.4 + 200	500 + ≥2[c]
			6.3 + 25	8 + 2[c]	50[18]	25	12.5	1.6 + 0.025	16 + 512
23	25	25	6.3 + 6.3	4 + 4	51[17]	200	>200	100 + 1.6	2 + >128[c]
24[1,d]	50	>200	25 + 0.4	2 + ≥512[c]				0.4 + 200	512 + ≥2[c]
			0.4 + 200	128 ≥2[c]	52	50	50	6.3 + 0.1	8 + 512
25	200	>200	0.4 + 200	512 + ≥2[c]				1.6 + 12.5	32 + 4
26[2]	50	>200	25 + 1.6	2 + ≥128[c]	53[a]	200	0.4	0.4 + 0.1	512 + 4
			0.4 + 200	128 + ≥2[c]	54[a]	100	>200	0.4 + 200	256 + ≥2[c]
27[3]	100	>200	0.4 + 200	256 + ≥2[c]	55	25	>200	0.4 + 200	128 + ≥2[c]
28[4]	100	>200	25 + 200	4 + ≥2[c]	56	200	>200	100 + 1.6	2 + ≥128[c]
29[5]	200	>200	100 + 1.6	2 + ≥128[c]				0.4 + 200	256 + ≥2[c]
			25 + 200	8 + ≥2[c]	57[15]	25	>200	0.4 + 200	64 + ≥2[c]
30[a]	25	100	0.4 + 0.1	64 + 1000	58	12.5	>200	0.4 + 200	32 + ≥2[c]

[a] Recent isolates from clinical cases.
[b] Indifferent.
[c] At least additive; possible synergy.
[d] Superscripts indicate number of same strain in first test.

were used in both the first and second sets of tests are compared in the lower part of Table 3 (section B). In the first run, 8 of the 18 strains were inhibited by 25 μg or less of ampicillin per ml, one was not inhibited by 200 μg of ampicillin per ml, and 9 were inhibited by 50 or 100 μg of each antibiotic per ml. In the second run, the strains were almost equally divided in their susceptibility to ampicillin among those inhibited by 25 μg or less per ml, those that required 50 or 100 μg/ml for inhibition, and those not inhibited by 100 μg/ml. Erythromycin, on the other hand, in concentrations of 25 μg or less per ml, inhibited only three of the 18 strains in the second run; the other 15 strains grew well in 100 μg/ml.

Effect of density of the culture. The growth of the strains used in the two sets of tests also differed as evidenced by numbers of CFU demonstrated at the time they were used in the susceptibility test. Only 3 of the 18 strains tested on both occasions achieved a smaller density in the second run; one of these strains is listed in Table 1 as no. 10 and 42, the second as no. 11 and 43, and the third as no. 18 and 50. The numbers of CFU in these cultures when used in the second run were one-half, one-third, and one-eighth of the corresponding numbers in the first. Two of these three strains were the only ones with which definite synergy was demonstrated in both tests. Of the other 15 strains, three had approximately the same number of CFU in both runs (one listed as 5 and 29, the second as 14 and 46, the third as 17 and

TABLE 2. *Summary and analysis of synergy of ampicillin (A) and erythromycin (E) against 40 strains of Nocardia asteroides*[a]

Analysis	Minimal inhibitory concn (μg/ml)		
	A	E	A + E
I. *Tests of strains not showing synergy*[b]			
Range	3.1->200	1.6->200	
Median	50	>200	
Geometric mean	57	160	
II. *Tests showing synergy*[c]			
Range	6.3->200	0.2->200[d]	0.4-25 + 0.025-25
Median	25	25	1.6 + 0.4
Geometric mean	32	10	2 + 0.8
Fold reduction			
Range			4-512 + 4->512
Median			16 + 8
Geometric mean			17.5 + >16

[a] Fifty-eight tests of 40 strains (18 tested on two separate occasions).

[b] Twenty-five additive and possible synergy plus three indifferent.

[c] Thirty strains, 34 combinations; only those showing fourfold or greater reductions in MIC of both antibiotics.

[d] MIC >200 μg/ml for only one strain; for the others it was ≤100 μg/ml.

51); the other strains had 3 to 30 times the number of CFU in the second test as they had in the first.

Effect of time of incubation. The first two studies thus differed both in some details of method and in the proportion of strains showing synergy by the definition used here. To determine whether the length of incubation was a major factor in determining the occurrence of synergy, a third study was carried out specifically for this purpose.

For this study, 36 strains which had grown to moderate density after 48 h in Mueller-Hinton broth were used. Plate counts of the cultures were not done in this test, but three of the strains failed to provide an adequate number of CFU and did not yield confluent growth on control plates of antibiotic-free agar. The other 33 strains showed adequate growth and were tested by the method described, except that both antibiotics were included at concentrations of 400 μg/ml, and MICs were recorded at 24, 48, and 72 h (each ±4). The results of all these tests are shown in Table 4, and some of the more relevant results are represented graphically in summary form in Fig. 1.

The lowest panel on the left in Fig. 1 shows clearly the marked increases in the MICs of erythromycin for all the strains between 24 and 48 h and the further increases for most of them after the additional 24 h of incubation. On the other hand, the MICs of ampicillin for the same strains, which were all much higher at 24 h, were increased only slightly but progressively with further incubation. An analysis of these results is shown in Table 5 which also lists the number of strains showing synergy at the three periods of incubation.

The top panel on the left side of the figure shows the marked reduction in the MICs of ampicillin in the presence of 6.3 μg of erythromycin per ml, and the progressive increases in the amounts of ampicillin required as incubation continued to 48 and 72 h. The other three panels show similarly striking but progressively smaller reductions in MICs of ampicillin in the presence of decreasing concentrations of erythromycin when compared with the corresponding values for ampicillin alone. The addition of as little as 0.4 μg of erythromycin per ml, which alone inhibited only three strains at 24 h and none at 48 and 72 h, increased the inhibitory effect of ampicillin for many strains. Concentrations of 25 and 50 μg of ampicillin per ml alone inhibited two and four strains, respectively, at 72 h; in the presence of 6.3 μg of erythromycin (which alone inhibited only one and two strains, respectively) per ml, the same amounts of ampicillin inhibited 13 and 23 strains.

Several new points emerged from the data shown in Table 4. After 24 h, synergy, as defined here, was noted with 28 (85%) of the strains.

TABLE 3. *Comparison of results of two sets of tests*

Analysis	Organisms tested					
	First set		Second set		Both[a]	
	No.	%	No.	%	No.	%
Combined action						
Synergy	15	65	15	43	30	52
Addition or possible synergy	6	26	19	54	25	43
Indifferent	2	9	1	3	3	5
Total tested	23	100	35	100	58	100
Susceptibility to each antibiotic alone						
A. All strains	23	100	35	100	58	100
Ampicillin						
≤25 μg/ml	11	48	17	48	28	48
50–100 μg/ml	11	48	9	26	20	35
≥200 μg/ml	1	4	9	26	10	17
Erythromycin						
≤25 μg/ml	12	52	12	34	24	42
50–100 μg/ml	11	48	2	6	13	22
≥200 μg/ml	0	0	21	60	21	36
B. Strains used in both sets	18	100	18	100	36	100
Ampicillin						
≤25 μg/ml	9	50	7	39	16	44
50–100 μg/ml	8	45	5	28	13	36
≥200 μg/ml	1	5	6	33	7	20
Erythromycin						
≤25 μg/ml	9	50	3	17	12	33
50–100 μg/ml	9	50	0	0	9	25
≥200 μg/ml	0	0	15	83	15	42

[a] Number of tests.

However, with each of the other five strains, some combination of ampicillin with erythromycin, for example, one-half of the MIC of one of the pair combined with one-fourth or one-eighth of the MIC of the other, inhibited growth. Moreover, three of the five strains were acted on synergistically at 48 and 72 h, the fourth (no. 9) showed synergy at 48 but not at 72 h, and the fifth (no. 45) did not show synergistic action at any of the three readings.

When the tests were read at 48 h, 31 strains (94%) were acted on synergistically and only 2 (6%) were not. However, at the 72-h readings, nine strains were recorded as showing less than synergistic responses to the combination. For eight of these nine strains, the MIC of ampicillin alone was 100–>400 μg/ml and that of erythromycin was >400 μg/ml; in combination, one-half of the MIC of ampicillin was inhibitory in the presence of 1/64th to 1/512th (mean 1/170th) of the MIC of erythromycin. For strain 45, the MIC of ampicillin alone was 3.1 μg/ml at 24 h and 6.3 μg/ml at 48 and 72 h, and the MICs of erythromycin alone for that strain were 0.4, 0.8, and 12.5 μg/ml at the successive readings; the combination of one-half of the MIC of ampicillin was inhibitory in the presence of 1/4th, 1/8th, and 1/32nd of the respective concentration of erythromycin.

Table 5 shows an analysis of the MICs of ampicillin and erythromycin when used individually against the 33 strains at each of the three readings in the third test. Though differing quantitatively for the 48- and 72-h readings from the first and second tests, respectively, the results and the relationships were quite similar. The numbers of strains acted on synergistically at each time interval are also shown in Table 5.

Analysis of the occurrence of synergy as observed in the third test is given in Table 6. The last column in part B of Table 6 shows that the mean reduction in concentration of ampicillin required for synergy (shown in part II of Table 6) was not significantly reduced as the length of incubation was increased; in contrast, the mean fold decreases in the median and mean concentrations of erythromycin in the synergistic combinations increased considerably and progressively with time.

DISCUSSION

Sulfonamides are generally considered the drugs of choice for the therapy of nocardiosis (4–7, 15); they are reported to be the most effective agents against experimental infections with *N. asteroides* in mice (5, 8, 12) and are generally active in vitro against clinical strains of this organism (3, 13). Nevertheless, the results of therapy and of in vitro tests for susceptibility have been variable (5, 6, 12) and do not correlate well (6, 10). Other antimicrobial agents are sometimes required when the sulfonamides are ineffective or cannot be tolerated, as was noted in the cases at the Boston City Hospital (1).

Sanford, Hatten, and Fordtran (9) found novobiocin and erythromycin to be the most active of eight antibacterial agents against 18 strains of *N. asteroides* which they tested; the former inhibited 16 of the strains in concentrations of <0.08 to 10 μg/ml and the latter inhibited 15 of them in 10 μg or less per ml. However, Black and McNellis (3), in a study of nine strains of *N. asteroides*, found only one of them susceptible to 3.12 μg of erythromycin per ml, another to 12.5 μg/ml, and seven required 25 to >100 μg/ml; the MIC of three strains of other species of *Nocardia* was 50 to 100 μg/ml. Larsen,

Diamond, and Collins (5) tested seven strains; the MIC of erythromycin for four was 0.6 to 12 μg/ml and for the other three it was >20 μg/ml. Wilson and Williams (13) tested 25 strains of *Nocardia* of which 11 (8 of human and 3 of animal sources) were *N. asteroides;* they found ampicillin to be the most active of the penicillins, but only 40% were inhibited by 5 to 25 μg/ml. Of the nine strains of *N. asteroides* reported by Black and McNellis (3), two were inhibited by ampicillin at 1.56 μg/ml, four by 12.5 or 25 μg/ml, and three by 50 to >100 μg/ml. The MIC of the three strains of other species of *Nocardia* was also 50 to >100 μg/ml. Orfanakis, Wilcox, and Smith (7) tested isolates of *N. asteroides* from four clinical cases; the MICs of ampicillin alone were 6.25 to 250 μg/ml, and for trisulfapyrimidines alone they ranged from 5 to 75 μg/ml. However, they demonstrated marked synergy when these agents were combined in "achievable concentrations," and therapy with both resulted in clinical improvement in all of the patients and arrest of the disease in two of them. The clinical and experimental studies of Murray and co-workers (6) did not show drug susceptibility tests to be an accurate guide to therapy. The marked differences in values reported from different laboratories and even from the same laboratory at different times further support this finding.

Confirming the observations of Wilson and Williams (13), we found ampicillin to be the most active of the currently available penicillins and cephalosporins, but only less than half of the strains tested were inhibited by 25 μg/ml in the first two tests, and the MICs of ampicillin in the third test were 50 to 200 μg/ml for most of the strains at 48 and 72 h. As previously shown by Sanford and co-workers (9), we also found erythromycin to be active in low concentrations against a large proportion of strains, but many of them required higher concentrations or were not inhibited by 100 μg of that antibiotic per ml (2). Considering differences in media, time of incubation (48 h in most instances), and the differences in the sources and maintenance of the organisms tested by different workers, the results have been reasonably consistent and compatible with those presented here with respect to the individual antibiotics.

In the second study (test no. 24 to 58 in Table 1), 20 of the 35 strains tested were not inhibited by 200 μg of erythromycin per ml alone but were inhibited by that concentration of erythromycin in the presence of 1/4th to less than 1/500th of the MIC of ampicillin alone. This was interpreted as being at least additive and possibly synergistic. In the third study, the two antibiotics were included at concentrations of 400 μg/ml, and 29 of the 33 strains tested were not inhibited at 72 h even by the higher concentration of erythromycin alone; nevertheless, only 8 of the 29 strains showed less than a synergistic effect of the combination with ampicillin. Strains which were inhibited by erythromycin at 200 μg/ml now showed synergy, as defined here, when compared in combination with low concentrations of ampicillin added.

The present studies thus confirm the favorable in vitro activity of ampicillin and erythromycin when used individually against most strains of *N. asteroides* and when the usual 48-h incubation period is used in the tests. It was further shown that, when combined, these two antibiotics exerted a definite synergistic effect on most of the strains, and their combined action was at least additive on almost all of the others.

The period of incubation was shown to have a major effect on the activity of erythromycin, and at 72 h most of the strains were not inhibited by 400 μg/ml, the highest concentration used in the tests. Runyon (8) also observed progressive (fourfold) increases in the MIC of several antibiotics against *Nocardia* during each of the first 3 days of incubation. In the present studies, inhibition by ampicillin appeared to be influenced much less than by erythromycin with increased time of incubation, although the MICs of ampicillin did increase somewhat for most strains during the second and third days. In the case of erythromycin, slower action and increased acidity of the medium during early phases of growth may have played a role in the increases in MIC of that antibiotic with increased time of incubation. However, further studies to define these parameters are indicated.

Certain other factors in the cultures also appeared to influence the combined action of ampicillin and erythromycin on individual strains. One was the susceptibility of the strains to erythromycin alone. Of the nine strains which were not acted on synergistically at 72 h in the third test, eight were not inhibited by 400 μg of erythromycin per ml. Neither the MIC of ampicillin alone nor the fold reduction in the concentration of ampicillin in the combination was greatly increased by the increased time of incubation. On the other hand, the fold reduction in concentrations of erythromycin which produced synergy in combination with ampicillin did show parallel changes as can be seen in the last column of Table 6, section II.

TABLE 4. *Effect of time of incubation on activity of ampicillin (A) and erythromycin (E), separately and combined, against 33 strains of Nocardia asteroides*[a]

Strain no.	24 h				48 h				>2 h			
	MIC (μg/ml)			Fold decrease (A + E)	MIC (μg/ml)			Fold decrease (A + E)	MIC (μg/ml)			Fold decrease (A + E)
	A	E	A + E		A	E	A + E		A	E	A + E	
1	50	1.6	12.5 + 0.4	4 + 4	100	>400	3.1 + 12.5	32 + ≥64	200	>400	50 + 6.3	4 + ≥128
							25 + 1.6	4 + ≥512				
2	100	1.6	25 + 0.8	4 + 2[b]	100	>400	0.4 + 12.5	256 + ≥64	200	>400	50 + 3.1	4 + ≥256
			50 + 0.4	2 + 4[b]			25 + 3.1	4 + ≥256				
3	50	12.5	3.1 + 3.1	16 + 4	100	>400	25 + 3.1	4 + ≥256	100	>400	25 + 6.3	4 + ≥128
			12.5 + 1.6	4 + 8			0.4 + 12.5	256 + ≥64				
4	100	1.6	25 + 0.4	4 + 4	100	25	6.3 + 6.3	16 + 4	200	>400	50 + 6.3	4 + ≥128
5	100	6.3	12.5 + 1.6	8 + 4	100	100	3.1 + 12.5	32 + 8	100	>400	50 + 6.3	2 + ≥128[b]
			25 + 0.4	4 + 16			25 + 6.3	4 + 16				
6	200	3.1	25 + 0.2	8 + 16	200	50	0.4 + 12.5	512 + 4	200	>400	0.4 + 12.5	512 + ≥64
							50 + 0.4	4 + 128			50 + 0.4	4 + ≥2,048
7	100	6.3	25 + 1.6	4 + 4	400	50	0.4 + 12.5	1024 + 4	400	>400	100 + 0.8	4 + ≥1,024
							100 + 0.4	4 + 128			50 + 6.3	8 + ≥128
9	100	1.6	50 + 0.4	2 + 4[b]	100	25	6.3 + 6.3	16 + 4	100	>400	50 + 3.1	2 + ≥256[b]
10	100	12.5	3.1 + 3.1	32 + 4	100	>400	50 + 6.3	2 + ≥128[b]	200	>400	100 + 3.1	2 + ≥256[b]
13	200	6.3	50 + 1.6	4 + 4	400	50	0.4 + 12.5	1024 + 4	>400	>400	100 + 12.5	≥4 + ≥64
							100 + 1.6	4 + 32				
14	400	12.5	3.1 + 3.1	128 + 4	>400	50	200 + 1.6	≥4 + 32	>400	>400	400 + 1.6	≥2 + ≥512[b]
			100 + 0.2	4 + 64								
15	200	12.5	3.1 + 3.1	64 + 4	200	50	12.5 + 12.5	16 + 4	400	>400	100 + 12.5	4 + ≥64
			50 + 0.2	4 + 64			50 + 6.3	4 + 8				
17	400	6.3	100 + 1.6	4 + 4	400	50	100 + 3.1	4 + 16	>400	>400	100 + 12.5	≥8 + ≥64
			50 + 0.8	8 + 8			50 + 6.3	8 + 8			200 + 3.1	≥4 + ≥256
18	200	25	3.1 + 3.1	64 + 8	200	100	25 + 12.5	4 + 8	200	>400	100 + 6.3	2 + ≥128[b]
							50 + 6.3	4 + 16				
20	200	3.1	50 + 0.2	4 + 16	200	25	50 + 6.3	4 + 4	400	>400	50 + 6.3	8 + ≥128
											100 + 1.6	4 + ≥512
21	100	1.6	25 + 0.4	4 + 4	200	25	12.5 + 3.1	16 + 8	400	50	50 + 6.3	8 + 8
							50 + 1.6	4 + 16			100 + 0.8	4 + 64
22	100	1.6	25 + 0.4	4 + 4	100	25	12.5 + 3.1	8 + 8	400	100	25 + 3.1	16 + 32
							25 + 1.6	4 + 16			100 + 0.2	4 + 512
25	100	3.1	25 + 0.4	4 + 8	100	25	25 + 3.1	4 + 8	100	>400	50 + 3.1	2 + ≥256[b]
28	200	3.1	25 + 0.4	8 + 8	200	12.5	25 + 3.1	8 + 4	200	400	25 + 12.5	8 + 32
											50 + 6.3	4 + 64
29	50	0.8	0.8 + 0.2	64 + 4	50	12.5	3.1 + 1.6	16 + 8	100	>400	12.5 + 0.8	8 + ≥1,024
			12.5 + 0.1	4 + 8			12.5 + 0.2	4 + 64			25 + 0.2	4 + ≥4,096
30	50	1.6	12.5 + 0.2	4 + 8	100	200	0.4 + 12.5	256 + 16	100	>400	25 + 6.3	4 + ≥128
							12.5 + 3.1	8 + 64				

31	12.5	0.8	6.3 + 0.1	2 + 8[b]	50	12.5	3.1 + 3.1	16 + 4	50	>400	6.3 + 6.3	8 + ≥128
32	25	0.4	6.3 + 0.1	4 + 4	50	12.5	3.1 + 1.6	16 + 8	50	>400	6.3 + 3.1	8 + ≥256
							6.3 + 0.2	8 + 64			12.5 + 0.8	4 + 1,024
33	50	0.4	6.3 + 0.1	8 + 4	50	1.6	6.3 + 0.2	8 + 8	100	>400	3.1 + 3.1	32 + ≥256
											25 + 0.1	4 + ≥8,192
35	200	12.5	25 + 3.1	8 + 4	200	>400	50 + 12.5	4 + ≥64	200	>400	100 + 12.5	2 + ≥64[b]
			50 + 1.6	4 + 8								
36	50	0.8	0.8 + 0.2	64 + 4	50	100	1.6 + 12.5	32 + 8	100	>400	12.5 + 3.1	8 + ≥256
			6.3 + 0.1	8 + 8			6.3 + 0.2	8 + 512			25 + 0.1	4 + ≥8,192
37	100	1.6	25 + 0.4	4 + 4	100	25	3.1 + 3.1	32 + 8	200	>400	12.5 + 3.1	16 + ≥256
							25 + 1.6	4 + 16			50 + 0.8	4 + ≥1,024
38	50	25	6.3 + 6.3	8 + 4	100	>400	0.4 + 12.5	256 + ≥64	200	>400	50 + 6.3	4 + ≥128
39	50	1.6	12.5 + 0.8	4 + 2[b]	50	25	6.3 + 6.3	8 + 4	200	>400	50 + 6.3	4 + ≥128
41	12.5	0.8	0.8 + 0.2	16 + 4	25	25	3.1 + 0.8	8 + 32	50	>400	0.8 + 12.5	64 + ≥64
			3.1 + 0.1	4 + 8			6.3 + 0.2	4 + 128			6.3 + 0.8	8 + ≥1,024
43	12.5	0.8	0.4 + 0.2	32 + 4	25	25	3.1 + 0.8	8 + 32	25	>400	3.1 + 3.1	8 + ≥256
			3.1 + 0.1	4 + 8			6.3 + 0.2	4 + 128			6.3 + 0.2	4 + ≥4,096
44	100	12.5	25 + 3.1	4 + 4	200	>400	25 + 12.5	8 + ≥64	200	>400	100 + 12.5	2 + ≥64[b]
							50 + 6.3	4 + ≥128				
45	3.1	0.4	0.4 + 0.2	8 + 2[b]	6.3	0.8	3.1 + 0.1	2 + 8[b]	6.3	12.5	3.1 + 0.4	2 + 32[b]
			1.6 + 0.1	2 + 4[b]								

[a] Strain numbers in this table bear no relation to the test numbers used in Table 1.
[b] More than additive.

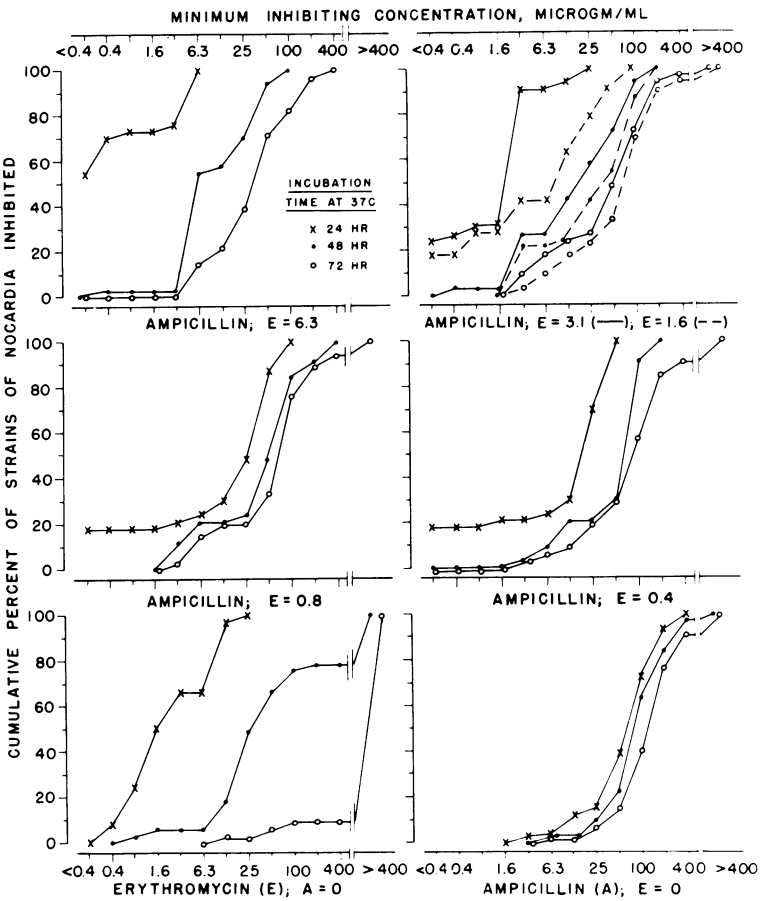

FIG. 1. *Nocardia asteroides. Activity of ampicillin and erythromycin separately and combined, and effect of time of incubation.*

TABLE 5. *Inhibitory effects of ampicillin (A) and erythromycin (E) on 33 strains of Nocardia asteroides after incubation for 24, 48, and 72 h at 37 C*

Antibiotic	MIC (μg/ml)	24 h		48 h		72 h	
		No.	%	No.	%	No.	%
Ampicillin	0.8–3.1	1	3	0	0	0	0
	6.3–25	4	12	3	9	1	3
	50–100	19	58	18	55	2	6
	200– >400	9	27	12	36	30	91
Erythromycin	≤0.4	3	9	0	0	0	0
	0.8–3.1	18	55	2	6	0	0
	6.3–25	12	36	14	43	1	3
	50–100	0	0	9	27	2	6
	200– >400	0	0	8	24	30	91
Combined action (A + E)							
Synergy[a]		28	85	31	94	24	73
No synergy[b]		5	15	2	6	9	27

[a] MIC of both A + E = one-fourth or less than MIC of each alone.

[b] By the same definition of synergy.

The size of the inoculum is another possible factor in the different results, but this was not clearly defined in the present studies. Preliminary tests with 10-fold increments, however, showed this more clearly. Also, when the inoculum was small or the cultures grew much more slowly, the MICs were markedly reduced so that synergy was sometimes difficult to demonstrate. The well-known tendency of *Nocardia* to clump may also have affected the results in the present studies, since some clumping in the wells of the replicator during the actual tests, as carried out, could not be avoided. These factors, too, need further study. Control plates with antibiotic-free agar were generally inoculated at the beginning and end of each test to assure proper growth and absence of contaminants from the inocula.

ACKNOWLEDGMENTS

This study was aided by Public Health Service grants 5ROI-AI-23 and 2TOI-AI-68 from the National Institute of Allergy and Infectious Diseases.

TABLE 6. *Summary and analysis of synergy[a] of ampicillin (A) and erythromycin (E) against 38 strains of Nocardia asteroides, and effect of time of incubation*

Analysis	Minimal inhibitory concn (μg/ml)			
	A	E	A + E	Fold decrease (A + E)
I. Tests of strains not showing synergy				
24 h (5 strains)				
Range (7)[b]	3.1-100	0.4-1.6	0.4-50 + 0.1-0.8	2-8 + 2-8
Median	50	1.6	12.5 + 0.2	2 + 4
Mean[c]	27	1.1	8.1 + 0.2	4.6 + 3.7
48 h (2 strains)				
Range (2)[b]	6.3-100	0.8->400	3.1-50 + 0.1-6.3	2 + 8-≥128
Mean[c]	25	≥25	12.5 + 0.8	2 + ≥32
72 h (9 strains)				
Range (9)[b]	6.3->400	12.5->400	3.1->400 + 0.1-12.5	≥2 + 32-≥512
Median	200	>400	100 + 3.1	2 + ≥64
Mean[c]	124	>400	62 + 3.1	2 + ≥128
II. Tests of strains showing synergy				
24 h (28 strains)				
Range (38)[b]	12.5-400	0.1-25	0.4-100 + 0.1-6.3	4-128 + 4-64
Median	100	3.1	12.5 + 0.4	8 + 4
Mean[c]	87	3.4	11 + 0.5	8 + 7
48 h (31 strains)				
Range (51)[b]	6.3->400	0.8->400	0.4-200 + 0.1-12.5	≥4-1,024 + 4-≥512
Median	100	50	12.5 + 3.1	8 + 16
Mean[c]	106	60	9.6 + 3.0	12 + 21
72 h (24 strains)				
Range (38)[b]	25->400	50->400	0.4-200 + 0.1-12.5	4-512 + 4-≥8,192
Median	200	>400	25 + 3.1	8 + ≥128
Mean[c]	174	>400	25 + 3.0	7 + ≥240

[a] MIC of both A + E = one-fourth or less when combined as compared to the individual MICs.
[b] Figures in parentheses indicate number of combinations.
[c] Geometric mean.

We are indebted to Ruth E. Gordon, Institute of Microbiology, Rutgers University, for supplying most of the strains of *Nocardia asteroides* used in this work.

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