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In Vitro Effects of Combination of Antituberculosis Drugs on Drug-Resistant Mycobacterial Strains**

Kyungsoo Kim, M.D.*

=국문초록=

各種 耐性抗酸性菌株들에 對한 混合抗結核劑의 *in vitro*에서의 效果

金 旻 守

National Jewish Hospital and Research Center Denver, Colorado 80206 U.S.A.

抗結核劑 kanamycin, ethambutol, ethionamide, cycloserine, 그리고 isoniazid 의 單獨 或은 併
합으로 *M. avium*, *M. intracellulare*, *M. scrofulaceum*, *M. fortuitum* 과 耐性人型結核菌에 對한
*in vitro*에서의 抗菌作用을 觀察하였다.

M. avium 과 *M. intracellulare* 는 3, 4 或은 5가지 混合藥品에 對하여 抑制作用을 나타내었다. 特
히 kanamycin+ethionamide+ethambutol 3가지 混合藥品에 對해서는 抑制作用이 높았으며, 顯著
한 藥品間의 相乘作用을 볼 수 있었다. 以外 다른 菌株에서도 어느程度 相乘作用을 볼 수 있었다.

Summary

The effects of kanamycin, ethambutol, ethionamide, cycloserine, and isoniazid, singly and in all combinations, on growth of *Mycobacterium avium*, *M. intracellulare*, *M. scrofulaceum*, *M. fortuitum*, and drugresistant *M. tuberculosis* were studied. Strains of the *M. avium M. intracellulare* group were inhibited by many of the 3-drug combinations, and by most of the 4-drug combinations, and by the 5-drug mixture. One triplet, kanamycin+ethionamide+ethambutol, is particularly effective and a synergistic relationship between the drugs is suggested. Variable results in regard to inhibition by the drug combinations were found

with the other strains tested.

Introduction

During the past several years a number of patients with infections due to *Mycobacterium intracellulare* and *M. avium* have been successfully treated with chemotherapy at National Jewish Hospital and Research Center. Most of these patients were ineligible for surgery due to pulmonary insufficiencies and the extent of their disease. Approximately 80% of them have achieved a quiescent status when given 4, 5, or 6 antituberculosis drugs simultaneously. The regimens employed usually included an injectable drug, and 4 or 5 oral medications. Since susceptibility tests indicated that most of the bacterial strains isolated were resistant *in viro* to the drugs employed clinically, the effects of combinations of these drugs on growth of the bacteria in the laboratory were studied. This report describes the results of these studies.

*National Jewish Hospital and Research Center, Denver, 80206 Present Address: PO Box 1834 Hagerstown, Md 21742 U.S.A.

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Methods and Materials

Bacteria: All of the strains employed were isolated from patients at this institution and were identified by the usual cultural and biochemical criteria¹¹. Serological typing was carried out W.B. Schaefer of this institution whenever possible.

Media and Drugs: Subcultures of the isolates were grown in 7H9 broth (Difco) at 37°C and stored at -90°C until used. Cohn 7H11 agar • (7H11 agar base, BBL; OADC enrichment, Hemo-line, Inc.) was used for the susceptibility tests. All drugs employed were sterilized by membrane filtration (0.22 μ pore size, Millipore) and added aseptically to cooled 7H11 agar prior to pouring of Petri dishes. Drugs used were isoniazid (INH, K & K Laboratories), p-aminosalicylic acid (PAS, J.T. Baker Chemical Co.), streptomycin (SM, Sigma Chemical Co.), kanamycin (KM, Bristol Laboratories), viomycin (VM, Pfizer Lederle Laboratories), capreomycin (CM, Eli Lilly & Co.), ethambutol (EMB, Lederle Laboratories) ethionamide (ETA, Ives Laboratories), cycloserine (CS, Eli Lilly & Co.), rifampin (RM, Dow Chemical Co.), and pyrazinamide (PZA, K & K Laboratories).

Susceptibility Tests: Drug susceptibility patterns of *M. intracellulare* and *M. avium* strains were determined in the standard manner¹². Results of both direct and indirect tests were used in the compilation of the data. Pyrazinamide susceptibility tests were performed by precisely following the method of Stottmeier et al.¹³. For drug combination studies the following drugs were added to 7H11 agar singly and in all possible combinations: KM (5 μ g/ml), ETA (5 μ g/ml), EMB (5 μ g/ml), INH (1.0 μ g/ml), and CS (30 μ g/ml). In some experiments RM (1.0 μ g/ml) was used instead of INH. After preparation of the plated media, dilutions of the appropriate bacterial subcultures were plated in triplicate on the media and incubated at 37°C in a 5% CO₂ atmosphere for 21 days. Results were evaluated by comparing the results on drug-containing media with amount of growth

on control plates at dilutions yielding 200 to 500 colonies per plate (+ + to + + + growth¹¹). During the course of the experiments the opaque colonial variants^{4,5,6} which spontaneously arise during subculturing of either *M. intracellulare* or *M. avium* strains were disregarded, and the data reflect only those results obtained with more drug resistant, more virulent transparent colony types.

For the preparation of survival curves, bacteria were inoculated into 7H9 broth in Ehlenmeyer flasks containing appropriate antituberculosis drugs. Incubation was at 37°C with vigorous shaking for aeration. Samples were taken at various time intervals and dilutions prepared in 7H9 broth. Surviving bacteria were determined by triplicate plate counts on 7H11 agar.

Results

Drug-Susceptibility Patterns of *M. intracellulare*-*M. avium* Strains: The results of direct and indirect susceptibility tests on strains of *M. intracellulare* and *M. avium* isolated from patients are shown in Table 1. As can be seen, about one-third of the strains tested are susceptible to the higher concentrations of CS, ETA, and RM. Only occasional strains are sensitive to any of the other antituberculosis drugs. Similar results have been obtained by other investigators^{7,8}.

Drug Combination Studies on Strains of *M. intracellulare*-*M. avium*: Table 2 illustrates the results of drug combination studies on two strains of *M. intracellulare* serotype VII. These strains of similar serotype were chosen because one strain came from a successfully treated patient, and the other from a patient who never converted to sputum negativity. Both strains were inhibited by most of the three, four, and five-drug combinations, while the single drug and most of the double-drug combinations had lesser effects. However, the ETA-KM (#15) combination appears to be relatively effective. Little difference between the "success" and "failure" strains can be seen.

Because of the inhibitory effects of the drug

Table 1. Drug Susceptibility Patterns of *M. Avium-M. Intracellulare* Strains

Drug-Conc., (μ g)	No. Tested	No. Susceptible	% Susceptible
INH-0.2	97	0	0
INH-1.0	94	1	1
INH-5.0	26	4	15
SM-2.0	95	0	0
SM-10.0	32	0	0
PAS-5.0	84	3	4
KM-5.0	94	2	2
KM-10.0	33	0	0
VM-5.0	94	1	1
VM-10.0	33	0	0
CM-10.0	95	5	5
CM-20.0	31	0	0
EMB-5.0	97	1	1
EMB-10.0	92	7	8
ETA-5.0	94	13	14
ETA-10.0	94	35	37
CS-20.0	35	4	11
CS-30.0	75	30	40
RM-1.0	22	1	1
RM-5.0	80	10	13
RM-10.0	80	31	39
PZA-20.0	22	0	0
PZA-50.0	22	0	0
PZA-100.0	22	0	0

combinations on the two above strains, an additional 7 strains of *M. intracellulare* and 2 strains of *M. avium* were studied (see Table 3). All of the strains tested were resistant to the individual drugs. Only four of the two-drug combinations inhibited any of the strains. Most of the three-drug mixtures inhibited a majority of the strains tested. Combination #22 (INH-CS-KM) was particularly ineffective, while #24 (EMB-ETA-KM) inhibited all of the strains tested. The four and five-drug mixtures were also inhibitory to most of the cultures.

Additional strains were tested for the effects of the EMB-ETA-KM and the INH-CS-KM mixtures. Ten of 15 resistant strains (to the single drugs)

Table 2. Effect of Drug Combinations on Two Staring of *M. Intracellulare* Serotype VI

Drug Combination	Therapy Success	Result Failure
1. Control	++*	++
2. INH-10 μ g	++	++
3. EMB-5.0 μ g	++	++
4. ETA-5.0 μ g	++	+
5. CS-30.0 μ g	+	++
6. KM-5.0 μ g	++	++
7. INH-EMB	+	+
8. INH-ETA	+	+
9. INH-CS	+	++
10. INH-KM	+	+
11. EMB-ETA	+	+
12. EMB-CS	+	+
13. EMB-KM	+	+
14. ETA-CS	+	+
15. ETA-KM	+6	+3
16. CS-KM	+	+
17. INH-EMB-ETA	+	+
18. INH-EMB-CS	0	+
19. INH-EMB-KM	0	0
20. INH-ETA-CS	0	0
21. INH-ETA-KM	0	0
22. INS-CS-KM	0	0
23. EMB-ETA-CS	0	0
24. EMB-ETA-KM	0	0
25. EMB-CS-KM	0	0
26. ETA-CS-KM	0	0
27. INH-EMB-ETA-CS	0	0
28. ETA-EMB-INH-KM	0	0
29. CS-KM-INH-ETA	0	0
30. CS-KM-INH-EMB	0	0
31. CS-KM-ETA-EMB	0	0
32. CS-KM-INH-ETA-EMB	0	0

*++=200~500 colonier per plate

+ =100~200 colonies per plate

<100 colonier=actual number

were susceptible to the former combination, none were sensitive to the latter.

Drug Combination Studies on Other Mycobacterial Strains: Because of the apparent effectiveness

Table 3. Summary of Drug Combination Studies on Seven (7) Strains of *M. Intracellulare* and Two (2) Strains of *M. Avium*

Drug Combination	Number Strains*
1. Control	0
2. INH-1.0 μ g	0
3. EMB-5.0 μ g	0
4. ETA-5.0 μ g	0
5. CS-30.0 μ g	0
6. KM-5.0 μ g	0
7. INH-EMB	0
8. INH-ETA	0
9. INH-CS	0
10. INH-KM	0
11. EMB-ETA	0
12. EMB-CS	2
13. EMB-KM	2
14. ETA-CS	1
15. ETA-KM	2
16. CS-KM	0
17. INH-EMB-ETA	6
18. INH-EMB-CS	7
19. INH-EMB-KM	8
20. INH-ETA-CS	7
21. INH-ETA-KM	8
22. INS-CS-KM	3
23. EMB-ETA-CS	7
24. EMB-ETA-KM	9
25. EMB-CS-KM	8
26. ETA-CS-KM	8
27. INH-EMB-ETA-CS	8
28. ETA-EMB-INH-KM	9
29. CS-KM-INH-ETA	9
30. CS-KM-INH-EMB	9
31. CS-KM-ETA-EMB	9
32. CS-KM-INH-ETA-EMB	9

* Inhibition is regarded as a 90% reduction in colonies as compared with the control plates

of the drug combinations of the drug-resistant *M. intracellulare*-*M. avium* strains, two strains of *M. scrofulaceum*, and one strain of *M. fortuitum* were also tested (see Table 4). The latter strain was not greatly affected by even the 5-drug mix-

Table 4. Effect of Drug Combinations on Three (3) Other Mycobacterial Strains

Drug Combination	<i>M. scrofulaceum</i>		<i>M. fortuitum</i>
	H.R.	A.M.	
1. Control	‡‡*	‡‡	‡‡
2. INH-1.0 g	‡‡	‡‡	+
3. EMB-5.0 g	‡‡	‡‡	+
4. ETA-5.0 g	‡‡	‡‡	‡‡
5. CS-30.0 g	+	‡‡	+
6. KM-50 g	‡‡	‡‡	‡‡
7. INH-EMB	NT	‡‡	+
8. INH-ETA	NT	‡‡	+
9. INS-CS	NT	‡‡	+
10. INH-KM	NT	‡‡	+
11. EMB-ETA	NT	‡‡	+
12. EMB-CS	NT	+40	+
13. EMB-KM	NT	‡‡	+
14. ETA-CS	NT	‡‡	+
15. ETA-KM	NT	‡‡	+
16. CS-KM	NT	‡‡	+
17. INH-EMB-ETA	‡‡	+25	‡‡
18. INH-EMB-CS	+	‡‡	‡‡
19. INH-EMB-KA	‡‡	+	+
20. INH-ETA-CS	‡‡	‡‡	+
21. INA-ETA-KM	0	‡‡	+
22. INH-CS-KM	+	‡‡	‡‡
23. EMB-ETA-CS	+	‡‡	‡‡
24. EMB-ETA-KM	0	+8	‡‡
25. EMB-CS-KM	+13	‡‡	‡‡
26. ETA-CS-KM	0	‡‡	‡‡
27. INH-EMB-ETA-CS	+	0	‡‡
28. ETA-EMB-INH-KM	0	+4	‡‡
29. CS-KM-INH-ETA	0	+	+
30. CS-KM-INH-EMB	+26	0	+
31. CS-KM-ETA-EMB	0	0	+
32. CS-KM-INH-ETA-EMB	0	0	‡‡

*‡‡=500~1,000 colonies per plate

‡‡=200~500 colonies per plate

+ =100~200 colonies per plate

<100 colonies=actual number

NT=not tested

ture. One of the *M. scrofulaceum* strains (H.R.) appears to be slightly more susceptible to the

Table 5. Effect of Drug Combinations on Two (2) Drug Resistant Strains of *M. Tuberculosis*

Durg Combination	Strain	
	A.S.	E.W.
1. Control	++*	+
2. RM-1.0 µg	++	+
3. EMB-5.0 µg	++	+
4. ETA-5.0 µg	++	+
5. CS-20.0 µg	++	+
6. KM-5.0 µg	++	+
7. RM-EMB	+	+
8. RM-ETA	+	+
9. RM-CS	+	+
10. RM-KM	+10	+
11. EMB-ETA	+	+
12. EMB-CS	+	+
13. EMB-KM	+	+
14. ETA-CS	++	+
15. ETA-KM	0	+
16. CS-KM	++	+
17. RM-EMB-ETA	0	+
18. RM-EMB-CS	+	+
19. RM-EMB-KM	0	+
20. RM-ETA-CS	+22	+
21. RM-ETA-KM	0	+
22. RM-CS-KM	+17	+
23. EMB-ETA-CS	0	+
24. EMB-ETA-KM	0	+3
25. EMB-CS-KM	+12	+
26. ETA-CS-KM	0	+
27. RM-EMB-ETA	+	+
27. RM-EMB-ETA-CS	0	+
28. ETA-EMB-RM-KM	0	+9
29. CS-KM-ETA	0	+
30. CS-KM-RM-EMB	0	+
31. CS-KM-ETA-EMB	0	+
32. CS-KM-RM-ETA-EMB	0	+

*++=200~500 colonies per plate
+=100~200 colonies per plate
<100=actual number colonies per plate

combinations than does strain A.M. The relative effectiveness of the EMB-ETA-KM triplet was

Fig. 1. Survival curve of a strain of *intracellulare* serotype VII exposed to EMB, ETA, and KM, singly, and in combination, in 7H9 brpth.

again observed.

Two highly drug-resistant strains of *M. tuberculosis* were tested in a similar manner. However, for these studies INH was replaced by RM (see Table 5). One of the strains (E.W.) was not greatly affected by any of the mixtures except #24 and #28. Strain A.S. gave results similar to most of the naturally drug-resistant *M. intracellulare*-*M. avium* strains.

Survival Curves Using The EMB-ETA-KM Mixture: Cultures of *M. intracellulare* serotype VII ("success" strain in Table 2) in 7H9 broth containing 5 µg KM/ml, EMB/ml, and 5 µg ET A/ml, singly and mixed together, were sampled at various times and surviving bacteria enumerated. Both ETA and EMB have low bactericidal properties; kanamycin reduced viability slightly. However, the 3 drugs in combination reduced viability by 2 logs in 24 hours and 4 logs in 96 hours.

Discussion

Multi-drug chemotherapy is routinely used in the treatment of *Mycobacterium tuberculosis* infections to prevent the emergence of drug-resistant mutants. Our rationale for treating other mycobacterial infections with 4, 5, or 6-drug regimens

was to achieve therapeutic results not obtainable with the use of the single drugs alone. Emergence of drug-resistant mutants was not a factor since most of the strains isolated were naturally drug-resistant. In other areas of infectious diseases, drug combinations are used with the hope that one drug will enhance the antibacterial activity of the second drug against a specific microorganism. Unfortunately, drug synergism is rare, usually unpredictable, and often quite specific for each bacterial strain⁹.

Our studies indicate that most of the strains of *M. intracellulare*-*M. avium* studied are inhibited by many 3-drug combinations and by most 4 and 5-drug mixtures of KM, ETA, CS, and INH *in vitro*. The results of the experiments indicate that the inhibitory effects are greater than additive or synergistic. This is further indicated by the survival curve experiment using the EMB-ETA-KM mixture. However, the synergistic relationship appears to involve all three drugs used simultaneously since much less effect was obtained using two-drug combinations of ETA, EMB, and KM. Studies are now in progress to investigate the efficacy of this three-drug regimen in treating mice infected with *M. intracellulare* strains.

Combination drug susceptibility tests were also carried out using strains of *M. tuberculosis* resistant to all eleven of the antituberculosis drugs currently available in the U.S. On the basis of the clinical laboratory susceptibility data, the treatment of such patients seems to be as hopeless as the therapy of patients infected with *M. intracellulare*-*M. avium* strains. Since quite different results were obtained in the drug combination with the two highly resistant *M. tuberculosis* strains, little can be said concerning the use of 4, 5, or 6 drug regimens in cases where the patient has acquired resistance to the drugs in question. It was found that the two *M. scrofulaceum* strains were significantly inhibited by the 4 and 5 drug mixtures and the data suggests that multi-drug regimens might be clinically effective.

These regimens do not appear to be indicated in treating *M. fortuitum* infections.

Several laboratories have investigated possible *in vitro* synergisms between pairs of antituberculosis drugs, usually INH plus one other drug. Goldstein, Eagle and LaCasse¹⁰ reported small degrees of synergy in two instances: (1) INH plus KM against an INH resistant strain of *M. tuberculosis*, and (2) INH plus ETA against a strain of *M. fortuitum*. Combinations of INH and four other antituberculosis agents had indifferent antibacterial effects. In another study, Hobby and Lenert¹¹ found that sub-inhibitory concentrations of RM increased the effectiveness of low concentrations of SM or INH against *M. tuberculosis* strain H37Rv. Lorian and Sabath¹² have found synergistic effects with several combinations of semi-synthetic penicillins for several strains of *M. tuberculosis*. The mechanism of this synergy appears to be a competitive inhibition of penicillinase by one penicillin with the result that the other penicillin is spared in activation¹³.

Synergistic effects of various penicillins in the treatment of murine tuberculosis have been demonstrated¹⁴. McCune et al.¹⁵ have used combinations of INH with SM, PAS, or PZA in *in vivo* studies. Only an INH-PZA combination showed an enhancement of activity as compared with the activity as compared with the activity of either drug alone. It was found that the drugs were capable of sterilizing the spleens of infected mice, but only if the administration of isoniazid preceded or was concurrent with administration of PZA. It has been suggested that INH is lethal to organisms growing at a normal pH and that PZA is slowly bactericidal to resting bacteria at a low pH in splenic tissue. Given together, or consecutively in the correct sequence, they are synergistic. Insufficient information is available concerning the modes of action of either EMB or ETA to postulate a mechanism for the apparent synergy of KM-ETA-EMB in our studies.

Stottmeier, Woodley, and Kubica¹⁶ have used a novel device for investigating effects of drug

combinations on mycobacteria. They placed broth cultures of mycobacteria into dialysis bags and dialyzed antimicrobials into and out of the bags. Two or three drug combinations of INH, methenamine, erythromycin, and oxacillin, and combinations of INH and rifampin were studied using this apparatus. Many of the three drug mixtures were found to reduce the microbial population in the dialysis bag within a week. The combinations were found to be bacteriostatic rather than bactericidal. The use of this apparatus overcomes one of the disadvantages inherent in our own study. In the agar plates containing the drug combinations the bacteria are in constant contact with high concentrations of all the drugs simultaneously. This would not be the situation in patients under treatment since the various drugs are absorbed and excreted at varying rates and the peak drug levels occur separately and not simultaneously.

At any rate, our results appear to offer a firm background for the treatment of *M. intracellulare*-*M. avium* infections with multi-drug regimens of the commonly used antituberculosis agents. Hopefully, studies such as this will also yield additional information concerning synergistic (or antagonistic) relationships between various antituberculosis drugs and allow clinicians to formulate antimycobacterial drug regimens for maximum clinical efficacy as well as to avoid emergence of drug resistance. Unfortunately, insufficient data concerning the mechanisms of action of antituberculosis drugs is available to allow prediction of possible synergistic drug combinations and one must rely on empirical observations for information of this type. However, the use of multi-drug susceptibility studies for general clinical usage is not indicated for the following reasons. First, most strains tested were found to be inhibited by the 4 or 5 drug combinations. Second, the test has little value in predicting the results of chemotherapy in an individual patient (see Table 2). And, third, a large labor burden would be placed upon any laboratory attempting to

evaluate the effects of the many combinations of the various antituberculosis drugs on an individual patient.

REFERENCES

- 1) Kubica GP, Dye WE: Laboratory methods for clinical and public health mycobacteriology. United States Public Health Service Pub. 1547, US Gov. Print Off, Washington DC, 1967
- 2) Cohn ML, Waggoner RF, McClatchy JK: The 7H11 medium for the cultivation of mycobacteria. *Amer Rev Resp Dis* 98:295, 1968
- 3) Stottmeier KD, Beam RE, Kubica GP: Determination of drug susceptibility of mycobacteria to pyrazinamide in 7H10 agar. *Amer Rev Respir Dis* 96:1072 1967
- 4) Vestal AL, Kubica GP: Differential colonial characteristics of mycobacteria on Middlebrook and Cohn 7H10 agar-base medium. *Amer Rev Respir Dis* 94:247, 1966
- 5) Schaefer WB, Davis CL, Cohn ML: Pathogenicity of transparent, opaque, and rough variants of *Mycobacterium avium* in chickens and mice. *Amer Rev Respir Dis*, 102:499, 1970
- 6) Ryneerson TK, Shrouts JS, Wolinsky E: Rifampin *In vitro* effect on atypical mycobacteria. *Amer Rev Respir Dis* 104:272, 1971
- 7) Carruthers KJM, Edwards RGB: 'Atypical' mycobacteria in Western Australia. *Amer Rev Respir Dis* 91:887, 1965
- 8) Johnston RN: The treatment of infection due to 'atypical' mycobacteria *Tubercle*. (Suppl) 50:88, 1969
- 9) Jawetz E: The use of combinations of antimicrobial drugs. *Ann Rev Pharmacol* 8:151, 1968
- 10) Goldstein E, Eagle MC, LaCasse ML: *In vitro* chemotherapeutic combinations against isoniazid-resistant *Mycobacterium tuberculosis* and *Mycobacterium fortuitum*. *Appl Microbiol* 22: 329, 1971
- 11) Hobby GL, Lenert TF: The action of rifampin alone and in combination with other antitu-

- berculosis drugs. Amer Rev Respir Dis 162:462, 1970
- 12) Lorian V, Sabath LD: The effect of some penicillins on *Mycobacterium tuberculosis*. Amer Rev Respir Dis 105:632, 1972
- 13) Kasik JE: Activity of some semisynthetic penicillins on *Mycobacterium tuberculosis*. in *Antimicrobial Agents and Chemotherapy 1964*, American Society for Microbiology, Ann Arbor Michigan p315, 1964
- 14) Kasik JE, Weber M, Winberg E, Barclay WR: The synergistic effect of dicloxacillin and penicillin G on murine tuberculosis. Amer Rev Respir Dis 94:260, 1966
- 15) McCune RM, Jr, Thompsett R, McDermott W: The fate of *Mycobacterium tuberculosis* in mouse tissues as determined by the microbial enumeration technique: II. The conversion of tuberculosis infection to the latent state by the administration of pyrazinamide and a companion drug. J Exp Med 104:763, 1956
- 16) Stottmeier KD, Woodley EL, Kubica GP: New approach for the evaluation of antimycobacterial drug combinations *in vitro* (the laboratory model man). Appl Microbiol 18:399, 1969
-