ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1979, p. 240-242
0066-4804/79/08-240/03$02.00/0

Vol. 16, No. 2

# Inhibition by Ethambutol of Mycolic Acid Transfer into the Cell Wall of *Mycobacterium smegmatis*

KUNI TAKAYAMA,[1]* EMMA L. ARMSTRONG,[1] KEITH A. KUNUGI,[1] AND JAMES O. KILBURN[2]

*Tuberculosis Research Laboratory, William S. Middleton Memorial Veterans Medical Center, Madison, Wisconsin 53705; Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706;[1] and Bureau of Laboratories, Center for Disease Control, Atlanta, Georgia 30333[2]*

Received for publication 29 May 1979

Ethambutol simultaneously inhibited the transfer (presumably via mycolyl acetyl trehalose) of mycolic acids into the cell wall and stimulated the synthesis of trehalose dimycolates of *Mycobacterium smegmatis*. Structural similarities of the drug and mycolyl acetyl trehalose suggested that competitive inhibition was involved.

Ethambutol (EMB) is an effective and specific antituberculosis drug used in combination with isoniazid in chemotherapeutic regimens (4, 5, 10, 11). Its mechanism of action is not known. Forbes et al. (3) proposed that EMB interferes with the role of polyamines and divalent cations in ribonucleic acid metabolism. Studies by Beggs and Andrews (2) on EMB binding by *Mycobacterium smegmatis* have weakened the argument for a divalent cation-required system as a specific EMB target site. Recent studies by Kilburn and Greenberg (6) showed that the apparent number of viable *M. smegmatis* cells rose sharply within the first 4 h of EMB exposure. These investigators theorized that the normally large clumps of cells disaggregated into smaller clusters, thus increasing their apparent viability. A reduction in the lipid contents of the cell wall might cause disaggregation of the cells. We have studied the early effects of EMB on the synthesis of various lipids in growing cultures of *M. smegmatis* and have found that EMB inhibits the transfer of newly synthesized mycolic acids into the cell wall.

A 200-μCi amount of [1-$^{14}$C]acetate (11.1 μCi/μmol) was added to a 250-ml culture of a susceptible strain of *M. smegmatis* absorbance at 650 nm of 0.02) which was grown in the modified Middlebrook 7H9 medium (Difco) on a rotary shaker at 37°C (6). After a 2-h incubation period, the culture was divided into two equal volumes; EMB was added to one culture to a final concentration of 3.0 μg/ml, and incubation was continued. At various time intervals, 10-ml samples were removed and added to a tube containing 250 mg of unlabeled cells and 1.0 mmol of KCN in 1.5 ml of water. A 5-ml quantity of ethanol was added to the cell suspension, mixed, and centrifuged at 500 × *g* for 10 min. The pellet was extracted three times with 3-ml portions of chloroform-methanol (2:1, vol/vol). This extraction was established to remove all of the lipids except the mycolic acids covalently linked to the cell wall (7). The extracted residue was saponified, and the radioactivity in the liberated $^{14}$C-mycolic acids was determined. This procedure measured the rate of transfer of mycolic acids into the cell wall. The synthesis rate of trehalose dimycolates was determined similarly except that the 10-ml samples were passed through a filter (0.45 μm; Millipore Corp.) to recover the cells. The filter disks containing the labeled cells were then extracted with 10 ml of chloroform-methanol (2:1, vol/vol) and analyzed for trehalose dimycolates as previously described (7, 8). The mycolic acids from the cell wall of *M. smegmatis* were purified and identified by nuclear magnetic resonance spectroscopy and mass spectrometry (7). Purified trehalose dimycolates derived from *Mycobacterium tuberculosis* strain Aoyama B (a generous gift from Edgar Ribi, Rocky Mountain Laboratory, Hamilton, Mont.) were used as the chromatographic standard. Silica Gel G thin-layer chromatographic separation of trehalose dimycolates was performed with the solvents chloroform–methanol–water (40:10:1, vol/vol/vol).

EMB did not affect the synthesis of mycolic acids in *M. smegmatis*, confirming the results of Winder et al. (13). However, Figure 1 shows that EMB inhibited the transfer of mycolic acids into the cell wall. This inhibition began 15 min after cellular exposure to the drug and complete inhibition was reached in about 90 min. The rate of synthesis of trehalose dimycolates was higher in the EMB-treated cells than the control cells after 15 min of exposure (Fig. 2). Thus, the inhibition of mycolic acid transfer into the cell wall and the increased rate of synthesis of trehalose dimycolates occurred simultaneously.

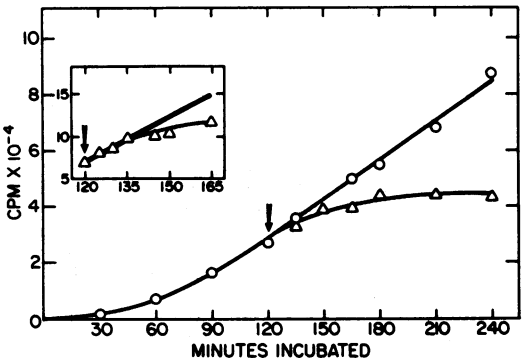



FIG. 1. *Effect of EMB on the transfer of $^{14}$C-mycolic acids into the cell wall of a susceptible strain of M. smegmatis. Symbols: ○, control cells; △, drug-treated cells. EMB (3.0 μg/ml) was added after 120 min of incubation (arrow). Inset examines the mycolate transfer reaction at the critical time intervals. Control curve (thick line) in the inset was established from a minimum of four points.*

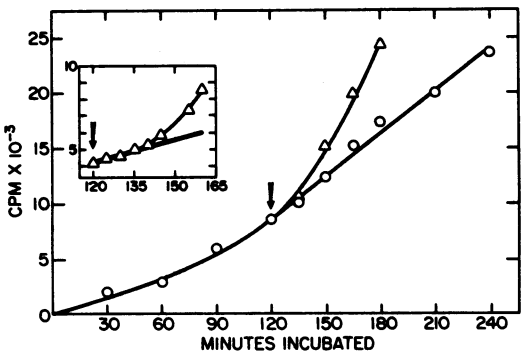



FIG. 2. *Effect of EMB on the synthesis of trehalose dimycolates in a susceptible strain of M. smegmatis. For description of the plot and inset, see the legend to Fig. 1.*

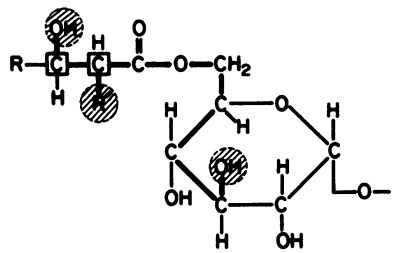
The resistant strain of *M. smegmatis* (sensitive to 50 μg of EMB per ml [6]) treated with 3.0 μg of EMB per ml showed a decrease of about 10% in the mycolic acid transfer reaction after 120 min of exposure (data not shown). When the resistant strain was exposed to 50 μg of EMB per ml, inhibition of mycolic acid transfer began after 30 to 40 min of exposure, and complete inhibition occurred after an additional 90 min.

The mycolic acids in the cells were prelabeled with [$^{14}$C]acetate, and then further synthesis of mycolic acids was stopped by adding isoniazid (10 μg/ml) and incubating for 15 min (9). The addition of EMB (3 μg/ml) to such a culture did not cause a decline in the prelabeled cell wall mycolic acids when assayed over the next 4-h period. This ruled out the possibility that the observed net inhibition of transfer of labeled mycolic acid into the cell wall might be due to the drug-enhanced hydrolysis of cell wall mycolic acids.

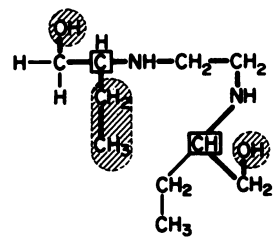
To our knowledge, this is the earliest response to EMB ever reported. If, as suggested by Takayama and Armstrong (8), mycolyl acetyl trehalose is on the pathway to mycolic acid transfer into the cell wall and this transfer is blocked by EMB, the pathway might be diverted to cause an accumulation of trehalose dimycolates. It is not possible at this time to relate these events to the observed disaggregation of cell clusters which occurs over the first 4 h of exposure to EMB (6) or to the more recent observation of EMB-enhanced phospholipid hydrolysis that occurs after 30 min of exposure (J. O. Kilburn, K. Takayama, J. Greenberg, and C. Callaway, unpublished data).

There are two possible explanations for the EMB inhibition of mycolic acid transfer into the cell wall of *M. smegmatis*. The drug may (i) inhibit the mycolic acid transfer reaction directly, or (ii) inhibit the synthesis of the cell wall mycolic acid acceptor. The primary action of EMB may involve one of these processes.

If EMB inhibits the mycolic acid transfer reaction directly, it could be competing with mycolyl acetyl trehalose for an active site on a proposed mycolate transfer enzyme (acyl trans-



TREHALOSE MONOMYCOLATE



ETHAMBUTOL

FIG. 3. *Structural similarity between trehalose monomycolate (mycolyl acetyl trehalose) and EMB as related to their binding groups. Only the partial structure of mycolyl acetyl trehalose is given. The asymmetric carbons (in blocks) have the dextro (+) configuration. The probable binding groups are indicated with darkened areas. R and R' are the alkyl side chains of the sizes $C_{50}$ and $C_{22}$, respectively.*

fer reaction). Structural similarities of these two compounds were revealed when we constructed the Prentice-Hall atomic stick models. Figure 3 shows these similarities. The functional groups involved in the binding of mycolyl acetyl trehalose might be the $\beta$-hydroxy and $\alpha$-alkyl groups of the mycolic acid moiety and the 3-hydroxy group of the trehalose moiety. Similar binding groups are shown in the EMB molecules. The asymmetric $\alpha$-carbon of mycolic acid is known to have a dextro (2R) configuration (1). The asymmetric carbons in the EMB must be in the dextro form for the drug to have tuberculocidal activity (10). Both drug and enzyme specificity could be derived from the configuration of the hydroxy and alkyl groups near the asymmetric centers of EMB and mycolic acid. Since a cell-free mycolate transfer enzyme system has yet to be isolated, such competitive inhibition by EMB cannot be demonstrated.

EMB represents yet another drug that affects the mycolic acid metabolism in mycobacteria. Three drugs that inhibit mycolic acid synthesis are isoniazid, ethionamide, and thiocarlide (9, 12, 13).

This investigation was supported in part by the Medical Research Service of the Veterans Administration and Public Health Service research grant AI-11297 from the National Institute of Allergy and Infectious Diseases.

We thank Cynthia Birch for her valuable editorial assistance on this manuscript.

## LITERATURE CITED

1. **Asselineau, C., G. Tocanne, and J. F. Tocanne.** 1970. Stéréochimie des acides mycoliques. Bull. Soc. Chim. Fr., p. 1455–1459.
2. **Beggs, W. H., and F. A. Andrews.** 1973. Nonspecific ionic inhibition of ethambutol binding by *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **4**: 115–119.
3. **Forbes, M., N. A. Kuck, and E. A. Peets.** 1965. Effect of ethambutol on nucleic acid metabolism in *Mycobacterium smegmatis* and its reversal by polyamines and divalent cations. J. Bacteriol. **89**:1299–1305.
4. **Kanther, R.** 1970. Myambutol: chemistry, pharmacology, and toxicity. Antibiot. Chemother. **16**:203–214.
5. **Karlson, A. G.** 1961. The *in vitro* activity of ethambutol (dextro-2,2'-[ethylenediimino]-di-1-butanol) against tubercle bacilli and other microorganisms. Am. Rev. Respir. Dis. **84**:905–906.
6. **Kilburn, J. O., and J. Greenberg.** 1977. Effect of ethambutol on the viability cell count in *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **11**:534–540.
7. **Takayama, K., and E. L. Armstrong.** 1976. Isolation, characterization, and function of 6-mycolyl-6' acetyltrehalose in the H37Ra strain of *Mycobacterium tuberculosis*. Biochemistry **15**:441–447.
8. **Takayama, K., and E. L. Armstrong.** 1977. Metabolic role of free mycolic acids in *Mycobacterium tuberculosis*. J. Bacteriol. **130**:569–570.
9. **Takayama, K., L. Wang, and H. L. David.** 1972. Effect of isoniazid on the in vivo mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **2**:29–35.
10. **Thomas, J. P., C. O. Baughn, R. G. Wilkinson, and R. G. Shepherd.** 1961. A new synthetic compound with antituberculous activity in mice: ethambutol (dextro-2,2'-[ethylenediimino]-di-1-butanol). Am. Rev. Respir. Dis. **83**:891–893.
11. **Wilson, T. M.** 1970. Clinical experience with ethambutol. Antibiot. Chemother. **16**:222–229.
12. **Winder, F. G., and P. B. Collins.** 1970. Inhibition by isoniazid of synthesis of mycolic acids in *Mycobacterium tuberculosis*. J. Gen. Microbiol. **63**:41–48.
13. **Winder, F. G., P. B. Collins, and D. Whelan.** 1971. Effects of ethionamide and isoxyl on mycolic acid synthesis in *Mycobacterium tuberculosis* BCG. J. Gen. Microbiol. **66**:379–380.