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Effects of Ethambutol on Accumulation and Secretion of Trehalose Mycolates and Free Mycolic Acid in *Mycobacterium smegmatis*

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We examined the early effects of ethambutol on the synthesis of trehalose monomycolate, trehalose dimycolate, and free mycolic acid in actively growing cells of *Mycobacterium smegmatis*. At about 1 min after the addition of 3.0 μg of ethambutol per ml, the cellular level of trehalose monomycolate began to increase over the control culture. This was followed 8 to 12 min later by the cellular increases in free mycolic acid and trehalose dimycolate over the control culture and the inhibition of incorporation of mycolic acid into the cell wall. Exposure of *M. smegmatis* to ethambutol for more than 30 min caused all of these lipids to leak out of the cells more rapidly than in the control cells. The mechanism by which ethambutol initiates these events is unknown, but these early imbalances in lipid synthesis may be responsible for the lethal action of this drug.

Ethambutol (EMB) is a primary drug used in treating tuberculosis. However, the mechanism of action of this valuable chemotherapeutic agent is not known (1). We have recently found that EMB affects the synthesis of cardiolipin and phosphatidylinositol mannosides and causes the leakage of phosphatidylethanolamine into the culture medium (5). We have also shown that the drug inhibits the incorporation of mycolic acid into the cell wall (7). We now report that EMB induces cellular accumulation and subsequent leakage of trehalose monomycolate (TM), trehalose dimycolate (TD), and free mycolic acid in *Mycobacterium smegmatis*.

MATERIALS AND METHODS

Bacteria and growth conditions. The strain of *M. smegmatis* used in this study was CDC 8 (derived from ATCC 607); it was grown in Middlebrook 7H-9 broth (Difco Laboratories, Detroit, Mich.). The albumin-dextrose enrichment was omitted, and 0.2% glycerol and 0.05% Tween 80 (Atlas Powder Co., Wilmington, Del.) were substituted. The cultures were grown at 37°C in a shaking water bath to an absorbance at 650 nm of 0.025 (approximately 2.5×10^6 colony-forming units per ml) before use.

Radioactive labeling and lipid extraction. To a culture grown to the desired absorbance was added 1.0 μCi of [1-^{14}C]acetate (58.7 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) per ml. The culture was divided into two equal volumes, and EMB was added to one to a final concentration of 3.0 μg/ml (minimum bactericidal concentration) and incubated at 37°C. In one experiment, cells were prelabeled with 1.0 μCi of [1-^{14}C]acetate per ml for 30 min, at which time a 100-fold excess of unlabeled acetate was added. The culture was divided, 3.0 μg of EMB per ml was added to one volume, and incubation was continued. At various times, 20-ml samples were transferred into tubes containing 1.0 ml of 1.0 M potassium cyanide to stop further incorporation of label into the lipids. The tubes were centrifuged at 27,000 × *g* for 15 min, and the upper 10-ml portions of the supernatants (growth medium) were carefully removed. The cell pellets were recovered separately, and the lipids were extracted by blending in a Vortex mixer in 4 ml of chloroform-methanol (2:1, vol/vol). The cell residue was separated from the chloroform-methanol by centrifuging at 27,000 × *g* for 15 min, and the solvent fraction was removed and evaporated to dryness. The remaining cell residue, containing the mycolic acid bound to the cell wall, was saponified overnight at 85°C in ethanolic potassium hydroxide. After the lipids were neutralized with 6 N hydrochloric acid, they were extracted with chloroform, and the chloroform layer was recovered after centrifuging at 500 × *g* for 10 min and evaporated to dryness.

The lipids secreted into the growth medium were extracted with an equal volume of chloroform after they were acidified with 1.0 ml of glacial acetic acid. The mixture was centrifuged at 500 × *g* for 10 min, and the chloroform layer was recovered and evaporated to dryness.

The extracted lipids from the cells, growth medium, and the saponified cell residue were redissolved in 300 μl of chloroform-methanol (2:1, vol/vol), and 50 μl was

spotted on silica gel plates.

Thin-layer chromatography. TM was separated on Silica Gel 60 (E. Merck AG, Darmstadt, Germany) by developing the plates in chloroform-methanol-water (65:25:4, vol/vol/vol). TD was separated on Silica Gel G (Analtech, Inc., Newark, Del.) with a solvent system of chloroform-methanol-water (90:10:1, vol/vol/vol). Mycolic acid was initially separated in the same solvent system as used for TD; however, because of comigration of two unidentified glycolipids, it was necessary to remove the area containing the mycolic acid, elute the lipids from the silica gel with chloroform-methanol-water (7:7:1, vol/vol/vol), concentrate the solvent, and run in a second solvent system of chloroform-methanol-concentrated ammonium hydroxide (95:5:1, vol/vol/vol). Detection of all lipid components was done with either iodine vapor or dichromate-sulfuric acid spray followed by charring in an oven at 120°C. The lipids were identified by using cochromatography with authentic standards. TM, TD, and mycolic acid were isolated from *M. smegmatis* and purified by the method of Takayama et al. (6, 8). The radioactive spots were scraped into vials containing 5 ml of Aquasol (New England Nuclear Corp., Boston, Mass.) and counted in a liquid scintillation spectrometer (Isocap/300; Searle Analytic, Des Plaines, Ill.).

RESULTS

Effects of EMB on TM and TD. Figure 1 shows the diphasic pattern of the synthesis of TM by *M. smegmatis* in the control cells. The initial rapid rate of synthesis of TM was followed by a steady state. The rate of leakage of newly synthesized TM into the culture medium in the control cells was very low (approximately 10% of the cellular accumulation). Incubation of *M. smegmatis* with EMB caused an accumulation of newly synthesized TM over the control cells. This began after 1 min of exposure to the drug (Fig. 1, inset) and continued through 60 min, at which time there was a 40% increase over the control cells. The drug also caused a rapid and large cellular leakage of TM after 30 min of exposure. After 90 min of exposure, the labeled TM found in the culture medium was 10 times that in the control medium.

There was an initial lag of about 8 min in the synthesis of TD by the control cells, which was followed by an approximate linear increase to 120 min of incubation (Fig. 2). Very little of the labeled TD was found in the culture medium of the cells incubated for 30 min, but further incubation caused the glycolipid to gradually leak out of the control cells and into the medium. The level of labeled TD in the medium was 24% of the cellular level after 120 min of incubation. Exposure of cells to EMB for 8 to 12 min caused a cellular accumulation of TD over the control level. The cellular TD content of *M. smegmatis* exposed to the drug for 60 min was 2.7 times

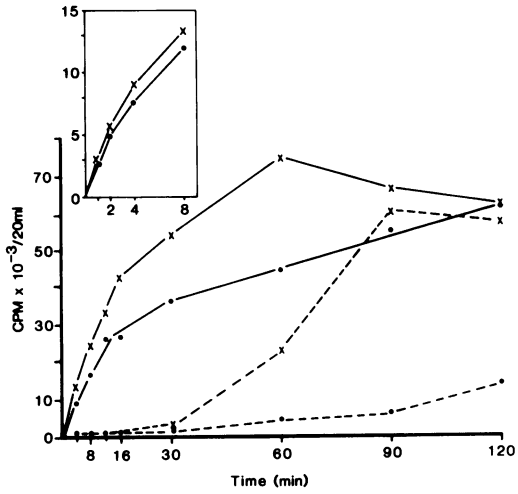

FIG. 1. *Effect of EMB on the synthesis and leakage of TM. EMB (3 μg/ml) and [1-^{14}C]acetate were added at time zero, and the amount of TM in the cells and growth medium was determined as described in the text. The onset of TM accumulation is shown in the inset. Symbols: ·——·, cellular control; ×——×, cellular EMB treated; · - - ·, growth medium control; ×- -×, growth medium EMB treated.*

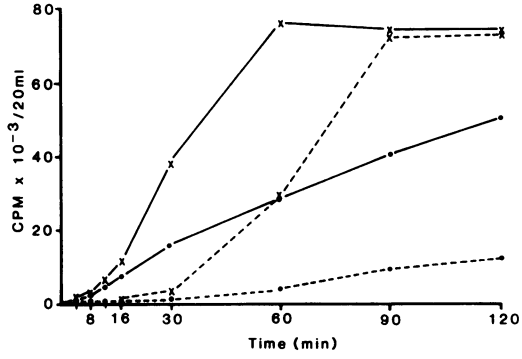

FIG. 2. *Accumulation and release of TD from EMB-treated M. smegmatis. Experimental procedures and symbols are the same as described in the legend to Fig. 1.*

that in the control cells. Exposure of the cells to EMB for over 30 min caused the TD to leak rapidly into the medium. The leakage of TD from cells exposed to the drug for 90 min was eight times that from the control cells.

Effects of EMB on free and cell-bound mycolic acids. Figure 3 shows the time course of synthesis of free mycolic acid in the control cells. A steady state of biosynthesis of this lipid was reached in the control cells after 30 min of [1-^{14}C]acetate incorporation. This was similar to the synthesis of TM. The amount of free mycolic acid synthesized was about 13 to 20% of the synthesis of either TM or TD. The rate of leak-

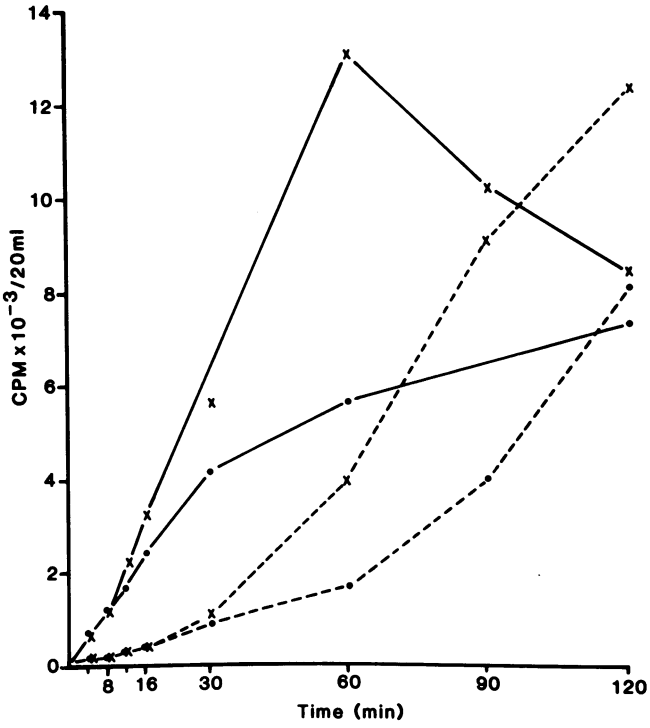

FIG. 3. *Effect of EMB on the cellular accumulation and leakage of free mycolic acid. Conditions and symbols are the same as described in the legend to Fig. 1.*

age of newly synthesized mycolic acid into the culture medium in the control cells rapidly increased with time until at 120 min of incubation approximately the same amount of this lipid was in the culture medium as was found in the cells. Incubation of *M. smegmatis* with EMB caused an accumulation of newly synthesized mycolic acid over the control cells. This began after 8 to 12 min of exposure to the drug. At 60 min of incubation, there was a 58% increase over the control cells. The drug also caused a rapid cellular leakage of mycolic acid beyond the control level. This began after 30 min of EMB exposure.

The inhibition in the incorporation of mycolic acid into the cell wall began when *M. smegmatis* was exposed to EMB for 8 to 12 min (Fig. 4). The onset of this inhibition was simultaneous with the increased cellular accumulation of TD and free mycolic acid.

Turnover of prelabeled TM. The cellular lipids of *M. smegmatis* were prelabeled by incubating the cells in the presence of [1-^{14}C]acetate for 30 min. A 100-fold excess of unlabeled acetate was then added to the culture to chase out the label in TM. The change in the labeled TM was followed over the next 60 min. The results showed that TM turned over rapidly and that EMB had no appreciable effect on this turnover rate (data not shown).

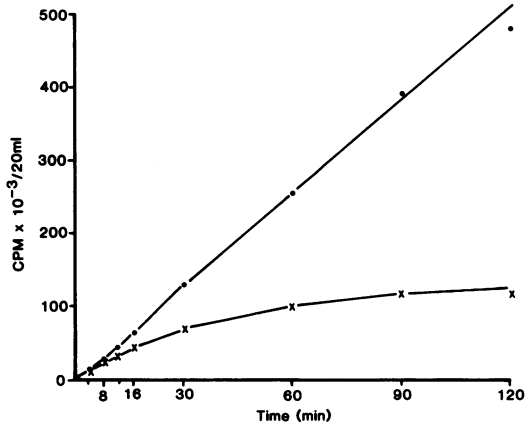

FIG. 4. *Time course of EMB inhibition of mycolic acid incorporation into the cell wall. EMB was added at time zero, and the amount of labeled mycolic acid released after saponification is shown. Symbols: ●, control mycolic acid; ×, EMB-treated mycolic acid.*

DISCUSSION

The present data show that after 1 min of exposure to EMB, *M. smegmatis* cells began to accumulate TM over the control cells. This is the earliest effect of EMB ever reported. This was closely followed, after 8 to 12 min, by the

simultaneous increases in the amount of free mycolic acid and TD over the control cells and the inhibition of mycolic acid bound to the cell wall. After 30 min of exposure to EMB, these lipids were no longer retained by the cells and appeared in large amounts in the growth medium. Previously, we showed that EMB induced the leakage of phosphatidylethanolamine after 30 min of drug exposure (5). This phospholipid may have been carried along with the other lipids. Since there was no change in absorbance at 650 nm during the experiments, leakage did not appear to be associated with bacterial lysis. Although *M. smegmatis* contains a large triglyceride pool, none of this material was found in the growth medium after EMB treatment (data not shown). This was additional evidence that bacterial lysis is not responsible for the leakage of TM, TD, and free mycolic acid. If, as suggested by Winder et al. (9), trehalose and the acyltrehaloses are synthesized near the cell surface, a buildup and subsequent dissociation from this area could occur without gross lysis. Horne and Tomasz have shown that penicillin induces the secretion of lipids in *Streptococcus sanguis* without cellular lysis (3). However, in contrast to EMB-treated *M. smegmatis*, there was no initial accumulation of lipids in this organism before their secretion. Beggs and Andrews showed that EMB binds almost instantaneously near the cell surface of *M. smegmatis* (2). Based upon these observations, it appears that the target site(s) for EMB is in the region of the cell surface.

The first observable effect of EMB on *M. smegmatis* is an increase in the cellular level of TM. What initiates this event is not known. TM may accumulate as a result of a blocking of the incorporation of mycolic acid into the cell wall by the drug. Although these two events do not appear to occur simultaneously, this could be due to limitations of the assay procedures. Alternatively, EMB may directly stimulate the synthesis of TM. The increased level of this glycolipid may interfere with the subsequent transfer of mycolic acid into the cell wall, resulting in a simultaneous buildup of free mycolic acid and TD. At present, it is not known how TM and TD are synthesized or what their extract functions are. However, the rapid turnover of these compounds has been described previously (6, 9). The turnover rate of prelabeled TM is not affected by EMB. Thus, the accumulation of this compound cannot be attributed to an inhibition of the enzyme(s) responsible for TM turnover.

EMB induces abnormally high levels of cellular and extracellular lipids, along with a decrease in cell wall and membrane lipids, followed by a decline in viable cells which begins 4 h after EMB exposure (4). We suggest, therefore, that this early imbalance in lipid synthesis is primarily responsible for the lethal action of this drug.

LITERATURE CITED

1. **Beggs, W. H.** 1979. Ethambutol, p. 43–66. *In* F. E. Hahn (ed.), Antibiotics, mechanism of action of antibacterial agents, vol. 5, part 1. Springer-Verlag, New York.
2. **Beggs, W. H., and F. A. Andrews.** 1974. Chemical characterization of ethambutol binding to *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **5:** 234–239.
3. **Horne, D., and A. Tomasz.** 1977. Tolerant response of *Streptococcus sanguis* to beta-lactams and other cell wall inhibitors. Antimicrob. Agents Chemother. **11:** 888–896.
4. **Kilburn, J. O., and J. Greenberg.** 1977. Effect of ethambutol on the viable cell count in *Mycobacterium smegmatis*. Antimicrob. Agents. Chemother. **11:**534–540.
5. **Kilburn, J. O., K. Takayama, E. L. Armstrong, and J. Greenberg.** 1981. Effects of ethambutol on phospholipid metabolism in *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **19:**346–348.
6. **Takayama, K., and E. L. Armstrong.** 1976. Isolation, characterization, and function of 6-mycolyl-6′-acetyltrehalose in the $H_{37}R_a$ strain of *Mycobacterium tuberculosis*. Biochemistry **15:**441–447.
7. **Takayama, K., E. L. Armstrong, K. A. Kunugi, and J. O. Kilburn.** 1979. Inhibition by ethambutol of mycolic acid transfer into the cell wall of *Mycobacterium smegmatis*. Antimicrob. Agents Chemother. **16:**240–242.
8. **Takayama, K., N. Qureshi, H. C. Jordi, and H. K. Schnoes.** 1979. Separation of homologous series of mycolic acids from *Mycobacterium tuberculosis* $H_{37}R_a$ by high performance liquid chromatography, p. 91–101. *In* G. L. Hawk (ed.), Biological/biomedical applications of liquid chromatography, vol. 10. Marcel Dekker, Inc., New York.
9. **Winder, F. G., J. J. Tighe, and P. J. Brennan.** 1972. Turnover of acylglucose, acyltrehalose and free trehalose during growth of *Mycobacterium smegmatis* on glucose. J. Gen. Microbiol. **73:**539–546.