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NOTES

Ethambutol Inhibition of Glucose Metabolism in Mycobacteria: a Possible Target of the Drug

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The addition of D-arabinose, D-galactose, D-glucosamine, or D-mannose to the growth medium of *Mycobacterium smegmatis* suppressed the inhibitory effects of ethambutol both on acetate labeling of cell wall-linked mycolic acids and on the increase in the delipidated cell dry weight. The addition of D-glucose or D-fructose had no effect. It is proposed that ethambutol inhibits an early step of glucose conversion into the monosaccharides used for the biosynthesis of structurally and biologically important cell wall polysaccharides: arabinogalactan, arabinomannan, and peptidoglycan.

Ethambutol (EMB) is an efficient antituberculosis molecule used in combination with other drugs (3). Moreover, there is renewed interest in EMB because of its activity against opportunistic pathogens, like *Mycobacterium avium* (4), which are resistant to conventional antituberculosis drugs and because of its ability to increase the susceptibility of this pathogen to other antimycobacterial drugs (5, 7, 13).

The mode of action of EMB has not yet been elucidated (for reviews, see references 7 and 16). The early inhibitory effects described for EMB concerned the synthesis of phospholipids (1, 8), the transfer of mycolic acids to cell wall-linked arabinogalactan (14), and the incorporation of labeled glucose into the arabinose-containing polysaccharides of the cell wall (15).

Inhibition of arabinogalactan synthesis would explain the inhibition of its acylation by mycolic acids, but the corresponding experiments have been performed with intact cells (14) and could be a secondary effect resulting from inhibition of some central metabolism.

Inhibition of cell wall-linked mycolic acid synthesis was tested in intact cells of *Mycobacterium smegmatis* ATCC 607 growing in Sauton's medium. EMB was added to an exponential-growth-phase culture (24 h of growth, ca. 1 mg of cell [dry weight] per ml medium) either at 1/10th of its MIC or at its MIC (5 μg/ml), and culture was continued for 1 h in the presence of [^{14}C]acetate (0.5 MBq/100 ml; 2 GBq/mmol; Amersham). Cells were collected by filtration and were extracted three times with $CHCl_3$-CH_3OH (2/1; vol/vol). Fatty acids were obtained by overnight saponification of extracted lipids and cell residues. Mycolic acids were obtained by precipitation of the saponified material with methanol; non-hydroxy fatty acids were recovered in the supernatant (2). All fractions were weighed and counted.

The effect of EMB on the arabinogalactan content of the cell wall was determined for cells that were delipidated with chloroform-methanol and then extracted with 70% ethanol to eliminate arabinomannan and mannan, which are not covalently linked to the wall (6). Cell residues were hydrolyzed (with 1 M trifluoroacetic acid at 110°C for 2 h), and the galactose content was determined by gas-liquid chromatography, with erythritol being added to the hydrolysis medium as a standard for quantification.

The cell-free system that incorporates acetate into mycolic acids was prepared and used as described previously (12) by isolating at the tops of centrifuge tubes the fluffy layer of cells from an exponential-growth-phase culture disrupted in a French pressure cell. The protein content of the fluffy layer was determined by the Lowry procedure as described previously (12). The assay medium did not contain magnesium, an ion known to reduce the activity of EMB. To test the drug effect, the cell-free system was preincubated for 30 min in the presence of 25 μg of EMB per ml (5× the MIC), and then [^{14}C]acetate was added for 90 min of incubation. Lipids were extracted as indicated above; lipids and cell residues were saponified, and total acids were isolated. Aliquots were counted, and total acids were analyzed as methyl esters by thin-layer chromatography (the solvent was CH_2Cl_2); radioactivity was determined on thin-layer chromatograms with a radioscanner (Berthold LB 2832) (10).

Reversal of the effects of EMB by monosaccharides that are constituents of cell wall glycoconjugates was tested by adding selected carbohydrates (5 mg/ml) to 24-h cultures (Sauton's medium) 1 h before simultaneously adding EMB (MIC, 5 μg/ml) and [^{14}C]acetate. Cells were harvested 1 h later to determine the labeling of cell wall-linked mycolic acids, as described above for assays with intact cells. Parallel experiments were performed without labeling to determine the dry weight of solvent-extracted cells.

For electron microscopy, cells grown in the presence of 5 μg of EMB per ml were observed at various times (1, 3, 6, 24, 48, and 96 h), after negative staining (2% phosphotung-

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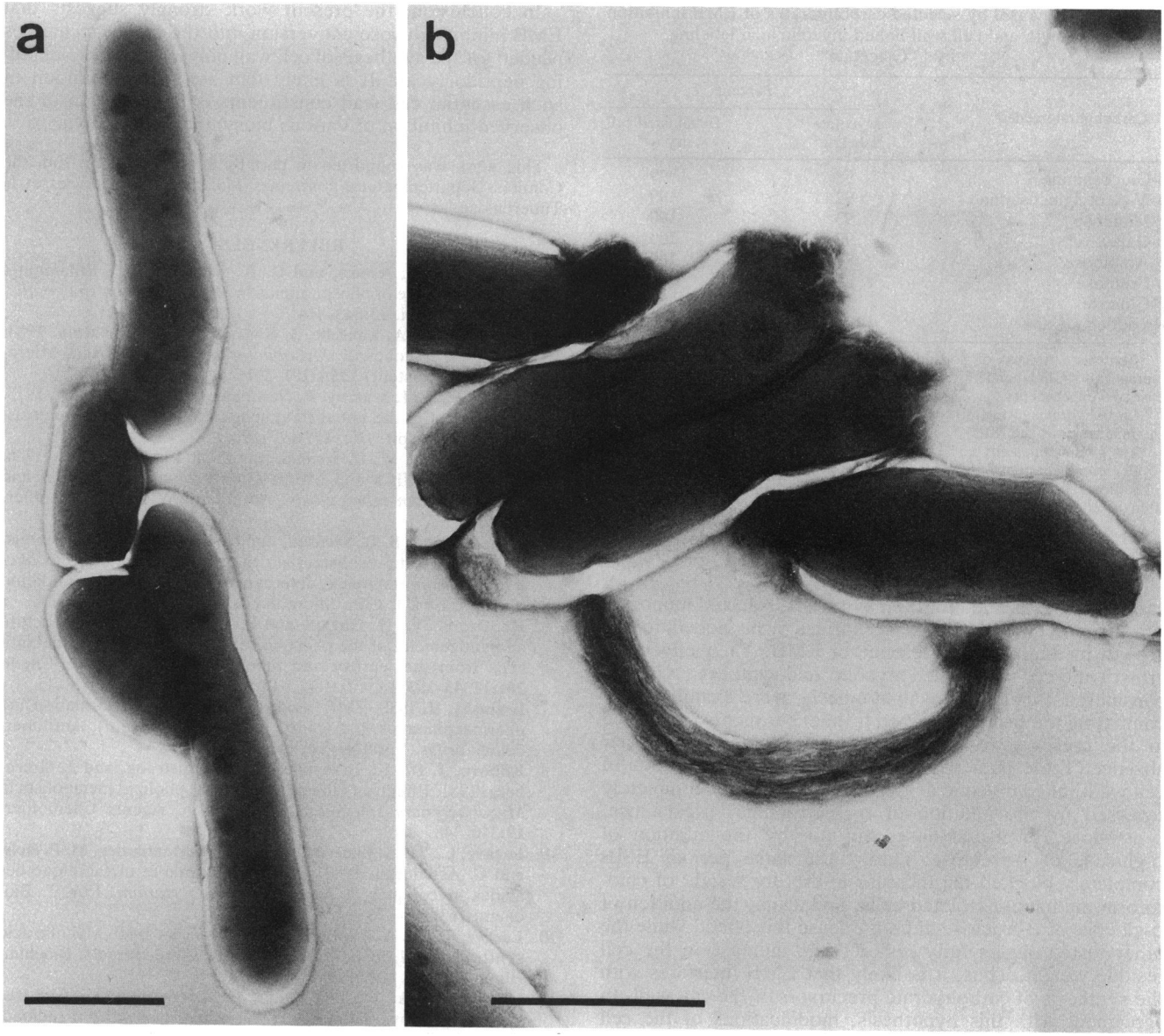

FIG. 1. Effect of EMB on *M. smegmatis* cells, as seen by negative staining. (a) Control culture; (b) cells from a 24-h culture grown for 6 h in the presence of 5 μg of EMB per ml (the MIC). Staining was with 2% phosphotungstic acid. Bars, 1 μm.

stic acid), by using a JEOL JEM 1200EX electron microscope.

There was strong inhibition of cell wall-linked mycolic acid labeling both at 1/10th the MIC and at the MIC (70 and 90% inhibitions, respectively), while no detectable inhibition of solvent-extractable mycolic acid labeling was observed. This resulted in a strong inhibition of total mycolic acid labeling, since cell wall-linked mycolic acids represent about 80% of mycolic acids (9). In addition, a concomitant inhibition of the labeling of non-hydroxy fatty acids (C_{12} to C_{24}) at both EMB concentrations (50 and 70%, respectively) was observed, a not previously reported observation which may explain the inhibition of phospholipid and mycolic acid synthesis, since C_{22} to C_{24} fatty acids are known to be precursors of mycolic acids (9).

To determine whether mycolic acid transfer to the cell wall was a direct target for EMB, a well-characterized insoluble cell-free system incorporating labeled acetate into cell wall-linked mycolic acids was used (10–12). No significant difference in the labeling of either cell wall-linked or extractable mycolic acids was noted in the presence of EMB (1.95 and 1.45 10^5 cpm/mg of protein, respectively) or in the absence of the drug (1.98 and 1.53 10^5 cpm/mg of protein, respectively). Thus, inhibition of mycolic synthesis by EMB could be an indirect effect, resulting from the action of the drug on a central metabolism.

Carbohydrate metabolism could be such a central target, since it has been shown that EMB inhibits [^{14}C]glucose incorporation in arabinogalactan (15). In agreement with this result, a 30% reduction in the arabinogalactan content of the cell wall was observed after 6 h in the presence of 5 μg of EMB per ml (the MIC) in the growth medium.

To see whether EMB inhibited either the monomer incorporation in polysaccharides or glucose conversion into the

TABLE 1. Reversal by selected carbohydrates of EMB inhibition of growth and cell wall-linked mycolic acid labeling by [^{14}C]acetate[a]

Carbohydrate added[b]	Percent[c]	
	Mycolate labeling[d]	Delipidated cell dry wt[e]
D-Glucosamine	98	99
D-*N*-Acetylglucosamine	97	99
D-Mannose	93	100
D-Galactose	92	100
D-Arabinose	86	100
D-Fructose	25	76
D-Glucose	17	80
No added sugar	15	75

[a] Controls consisted of no added EMB. Results without sugars are the averages of six independent experiments; other values are the averages of at least two experiments.
[b] Carbohydrates (5 mg/ml) were added 1 h before the addition of EMB (at 5 μg/ml, which is the MIC).
[c] The given values are ±5%.
[d] Percentage of control labeling (1 h of labeling).
[e] Ratio of assay/control (percent) of solvent-extracted cell dry weight at 1 h after the addition of EMB.

carbohydrates needed for their synthesis, selected monosaccharide precursors of polysaccharides were added to the growth medium in the presence of EMB. Their effects on growth and cell wall-linked mycolic acid synthesis, which is considered a marker of wall synthesis, were examined. By comparing the results obtained from experiments performed in the presence of sugars with those obtained in their absence (Table 1), it appeared that the drastic inhibition of cell wall-linked mycolic acid labeling was nearly completely reversed by the addition of D-glucosamine, D-galactose, D-mannose, or D-arabinose, but not by the addition of D-glucose or D-fructose. During the same period, EMB completely blocked the increase in the dry weight of chloroform-methanol-extracted cells, and again, the addition of D-glucose or D-fructose did not release this effect, while the other tested sugars fully restored the increase in the cell residue weight. Thus, it is likely that EMB interferes with the synthesis of carbohydrate precursors of the cell wall. In agreement with this hypothesis, modifications of the cell aspect were observed on micrographs of cells grown for 6 h in the presence of 5 μg of EMB per ml (the MIC) (Fig. 1), while lysis was detected on growth curves only 3 to 4 days after the addition of EMB.

Because the inhibitory effects of EMB on the synthesis of some cell wall constituents were reversed by adding sugars used to synthesize cell wall polymers, it is suggested that EMB inhibited glucose conversion into the carbohydrates needed for the synthesis of the glycoconjugates which are major constituents of the mycobacterial cell wall, namely, mycolylarabinogalactan, lipoarabinomannan, and peptidoglycan. Inhibition of such a central metabolism could trigger inhibition of other biosynthetic pathways, e.g., fatty acid synthesis, through general control mechanisms and could be at the origin of the variety of effects described for EMB. However, it is likely that the most deleterious effect of the drug is the inhibition of the synthesis of peptidoglycan precursors, and this would suffice to explain the antimycobacterial activity of EMB.

In conclusion, the present work strongly suggests that EMB inhibits glucose conversion into the monosaccharides needed for the synthesis of cell wall polysaccharides, including peptidoglycan. It is likely that synthesis inhibition of such essential cell wall constituents is at the origin of the observed inhibition of various biosynthetic pathways.

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