SEPARATION OF THE MIXTURE OF TREHALOSE 6,6'-DIMYCOLATES COMPRISING THE MYCOBACTERIAL GLYCOLIPID FRACTION, "P3"

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Summary: Ultra-purified trehalose dimycolate, "P3", an agent with potent host-reactive and immunological properties, including the ability to enhance tumor immune responses, was isolated from various strains of *Mycobacterium tuberculosis, M. bovis, M. avium,* or *M. phlei* and subjected to per-trimethylsilylation to permit chromatographic resolution of trehalose dimycolates containing different pairs of mycolic acids. Samples of trimethylsilylated P3 from virulent strains of human and bovine tubercle bacilli were resolved into six different components based on pairs of α, β, and γ-mycolic acids, whereas P3 from avirulent or attenuated strains contained fewer components due to the absence of detectable β-mycolic acid-containing diesters. The virulence of pathogenic mycobacteria may depend to a significant extent upon the presence or absence of a given component.

INTRODUCTION

A toxic lipid, designated "cord factor", obtained by superficial extraction of "cord-forming" young cultures of tubercle bacilli was first reported by Bloch (1). It was later isolated in larger quantities from mycobacterial waxes which had been extracted according to the procedures of Anderson (2,3,4), and was eventually characterized as α-D-trehalose 6,6'-dimycolate (5-9).

In previous reports from this laboratory, we described the fraction "P3" which corresponded to cord factor from which components other than trehalose mycolates had been removed by microparticulate silica gel chromatography (10, 11). Analysis of P3 by centrifugal chromatography or thin-layer chromatography portrayed only a single component (12, 13). However, when P3 was saponified,

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at least three mycolic acids were liberated (10). In earlier studies of mass spectra, Adam *et al.* (14) noted that cord factor samples from *Mycobacterium tuberculosis*, strain Peurois, and *M. bovis*, strain BCG, appeared to contain three and two types of mycolic acid, respectively, but the arrangements of the mycolic acids could not be ascertained, because no molecular peaks were detected. It was not unexpected that cord factor or P3 should contain more than one kind of mycolic acid, because several kinds are known to be present in the mycobacterial waxes, as both free acids and saponifiable esters (6,7,15,16,17). The presence of more than one kind of mycolic acid suggested that cord factor and P3 are mixtures of trehalose dimycolates containing different pairs of mycolic acids, but they were not resolvable by conventional chromatographic means. Recently, Promé *et al.* (18) applied a different approach to the study of cord factor from *M. phlei*: trimethylsilyl derivatization permitted chromatographic separation of three different symmetric and asymmetric trehalose di-phlei-mycolates, according to the polarity of the constituent mycolic acid residues.

In the present work, P3 fractions from various strains of mycobacteria were subjected to trimethylsilylation to permit chromatographic resolution of trehalose dimycolates containing various combinations of mycolic acids. P3 from virulent strains of human and bovine tubercle bacilli contained six different dimycolates, whereas the attenuated bovine strain, BCG, yielded three different dimycolates. Trimethylsilylated P3 samples from *M. avium* and *M. phlei* also displayed chromatographic patterns suggesting multiple components.

MATERIALS AND METHODS

Isolation of P3 (Trehalose Dimycolate). Samples of P3 were isolated from extracts of mycobacterial whole cells using pressure elution chromatography on columns of microparticulate silica according to previously described procedures (10). Cells of *M. tuberculosis*, strain Aoyama B, and *M. bovis*, strain BCG 1173 P2 (Pasteur Institute) were grown at the Rocky Mountain Laboratory (11). Cells of *M. bovis*, strain AN-5, and *M. avium*, strain D-4, were kindly supplied by Dr. R. Dale Angus, Veterinary Services Laboratory, Ames, Iowa 50010, and cells of *M. phlei*, strain 110, by Dr. Werner Brehmer, Tuberculosis Research Laboratory, Robert Koch-Institut, Berlin, West Germany. P3 from *M. tuberculosis*, strain Peurois, was obtained by chromatographing an unrefined cord factor preparation

extracted according to classical methods at Institut de Chimie des Substances Naturelles, CNRS, 91190 Gif sur Yvette, France.

Analysis of samples of P3, according to newly defined criteria using microparticulate silica gel chromatography, demonstrated the absence of polar polymeric material, trehalose monomycolates, free mycolic acids, or other detectable contaminants (19).

Formation of Trimethylsilyl (TMS)-P3. A 2- to 5-mg sample of the glycolipid was dissolved in 0.5 ml pyridine-hexamethyldisilazane-trimethylchlorosilane (9:3:1 v/v) ("Sil-Prep" reagent, Applied Science Laboratories, P. O. Box 440, State College, PA 16801) in a tightly capped tube and allowed to stand for about three hours at room temperature. The reagents were then evaporated under a stream of nitrogen, 1 ml of distilled water was added to the residue, and the mixture extracted twice with 1 ml of hexane. The hexanic extracts were pooled, dried with sodium sulfate, filtered, and evaporated to dryness. Infrared spectra revealed no remaining free hydroxyl groups.

Thin-layer Chromatography. Thin-layer chromatography (TLC) was performed with precoated, 20x20-cm plates of "Anasil-G", 0.25 mm layer thickness (Analabs, 80 Republic Drive, North Haven, CT 06473. The plate was spotted with approximately 100 μg of each sample dissolved in hexane (5 to 10 μl). TMS-P3 was developed with petroleum ether (b.p. 38 - 50°)-benzene (1:1 v/v), and methyl esters of mycolic acids were developed with petroleum ether-ether (85:15 v/v). To visualize the separated components, the plate was sprayed with 50% sulfuric acid and then heated at 150°.

Fractionation of TMS-P3 by Preparative TLC. TMS-P3 (2 to 6 mg) was applied as a streak on a 20x20-cm plate of Anasil G (prescored at 5 cm intervals; 0.25 mm layer thickness), and then developed in petroleum ether-benzene (1:1 v/v). The plate was fractured along the score lines, and the edge sections were subjected to sulfuric acid-charring to visualize the zones of separated TMS-P3 components. The silica was removed from the corresponding positions on the uncharred segments of the plate and extracted four times with 5 ml ether. Fractions thus obtained were examined for homogeneity by TLC (petroleum ether-benzene 1:1), rechromatographed if necessary by repeating the above procedure.

Transesterification of TMS-P3 or P3. Fractions of TMS-P3, P3 or other mycolic acid esters in benzene solution (1 to 5 mg/0.5 ml) were mixed with 1.0 ml 0.5 N methanolic sodium methoxide (Applied Science Laboratories) and then heated in a sealed tube for 30 min at 75°. After cooling, 1.5 ml distilled water was added, and the mixture extracted twice with 1.5 ml volumes of ether. The ether extracts were pooled, washed twice with 1.5 ml volumes of distilled water, dried with sodium sulfate, filtered, and the solvents evaporated.

TLC of Methyl Mycolates. Transesterification products of TMS-P3 and P3 were chromatographically compared (developing solvent: petroleum ether-ether 85:15 v/v) with methyl esters of authentic "α" (dicyclopropane)-, "β" (methoxy, cyclopropane)-, and "γ" (keto, cyclopropane)-mycolic acids from *M. tuberculosis*, strain Brévannes (17). Exposure of the mycolates to alkaline media as in transesterification with sodium methoxide or saponification with potassium hydroxide, resulted in the partial formation of the diastereomers "pre-α", "pre-β", and "pre-γ" (20). The diasteromers migrated faster than the respective native forms (hence the designation "pre-"), thus identification of each mycolate was based on the presence of two spots on the chromatogram, one containing the native mycolate and the other the "pre-" diastereomer.

RESULTS AND DISCUSSION

Samples of trimethysilylated P3 from pathogenic *M. tuberculosis*, strains

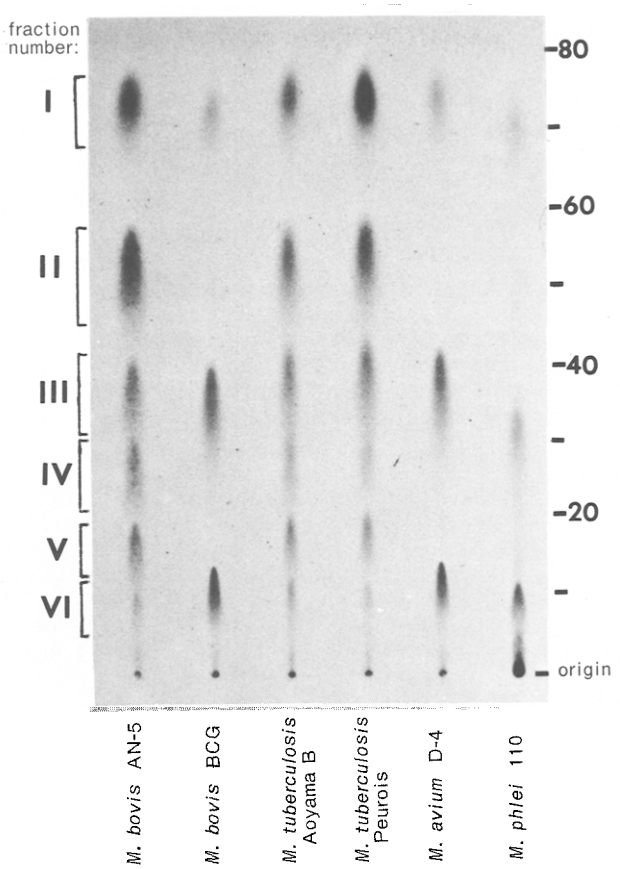

Figure 1. Thin-layer chromatograms of trimethylsilylated (TMS)-P3 from various strains of mycobacteria, showing the resolved trehalose dimycolate constituents. Developing solvent: petroleum ether-benzene (1:1 v/v). Single components from *M. bovis*, strain AN-5, (fractions I, II, III, IV, V, and VI) and from *M. bovis*, strain BCG (fractions I, III, and VI) were isolated by preparative thin-layer chromatography. The components I-VI of *M. bovis* AN-5 were found to contain α, α+β, α+γ, β, β+γ, and γ-mycolic acids, respectively, and the components I, III, and VI of BCG contained α, α+γ, and γ-mycolic acids, respectively (Fig. 2).

Aoyama B and Peurois, and *M. bovis*, strain AN-5, were resolved into similar patterns of six components each by thin-layer chromatography (Fig. 1). Each of the six components of *M. bovis* AN-5 was isolated by preparative thin-layer chromatography and then analyzed for its mycolic constituents (Fig. 2a). It was found that components I, IV and VI contained individual types of mycolic acids, corresponding, respectively, to α, β, and γ-mycolic acids of *M. tuberculosis*,

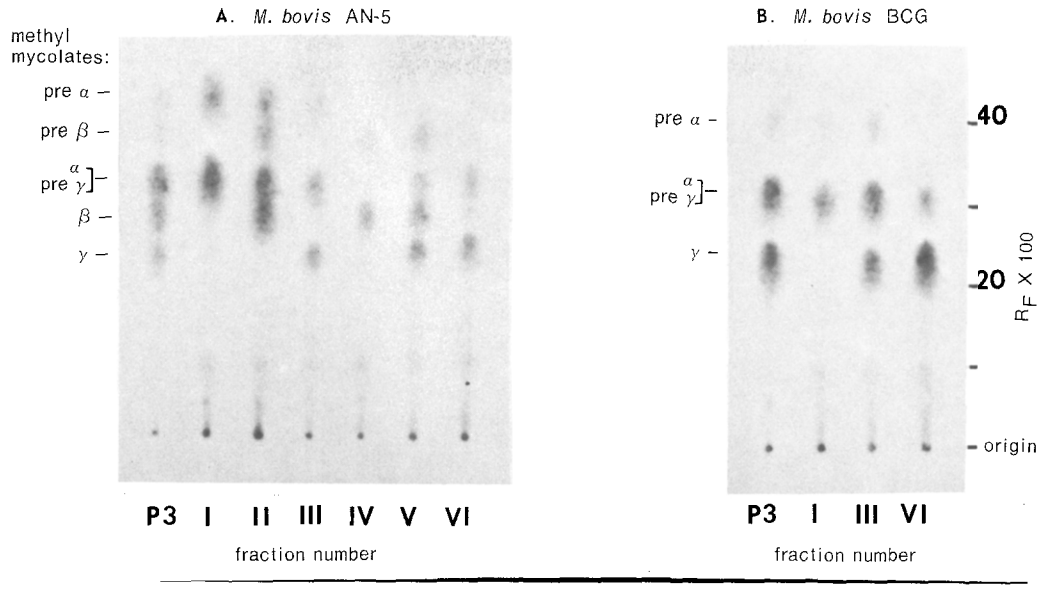

Figure 2. Thin-layer chromatograms of mycolic acid methyl esters liberated by transesterification of the individual fractions of TMS-P3 from strains AN-5 and BCG of *M. bovis* (Fig. 1). Developing solvent: petroleum ether-ether (85:15 v/v). Mycolic acids were identified by comparison with standards of previously characterized α, β, and γ-mycolic acid methyl esters from *M. tuberculosis*, strain Brévannes. Pre-α, pre-β, and pre-γ are diastereomers of the respective mycolates formed by exposure to alkali in the transesterification step. In this solvent system, α and pre-γ were not resolved.

strain Brévannes (see Materials and Methods section). Components II, III and V contained pairs of α+β, α+γ, and β+γ mycolic acids, respectively, in approximately equal proportions. If each of the above components represented a 6,6'-dimycolate of trehalose, we should conclude that P3 from *M. bovis* AN-5 was a mixture of three symmetric diesters (αα, ββ, and γγ) plus three asymmetric diesters (αβ, αγ, and βγ). Similar distributions of mycolic acids should be expected in the P3 of strains Aoyama B and Peurois for which nearly identical chromatographic patterns of TMS-P3 were obtained (Fig. 1). However, it may be that α, β, and γ-mycolic acids are not necessarily identical from one strain to another. It is likely that "α", "β", and "γ" should refer to classes of homologous mycolic acids containing particular functional groups, but which may vary according to the number of carbon units in the hydrocarbon chains (6,14,16). It is unlikely that variations

such as small differences in hydrocarbon chain length would be noticed in the present chromatographic systems.

In contrast to TMS-P3 of *M. bovis* AN-5, which contained six components, TMS-P3 from the attenuated strain of *M. bovis*, BCG, yielded merely three components (I, III, and VI, Fig. 1). When the respective transesterification products were chromatographed, similar mycolic acid constituents were found (α, $\alpha+\gamma$, and γ from fractions I, III and VI, respectively, Fig. 2b). In P3 prepared from BCG we noted the absence of all β-mycolic acid-containing components ($\alpha\beta$, $\beta\beta$, and $\beta\gamma$ or II, IV and V, respectively). Adam *et al.* (14) likewise identified only two mycolic acids in the cord factor of BCG, one with two cyclopropane rings and the other with a single cyclopropane ring plus a ketone in the meroaldehyde fragments of the respective mycolates, which correspond to α- and γ-mycolic acids described by Toubiana *et al.* (17).

The P3 of *M. avium* D-4 which, like BCG, is of limited virulence to man, similarly appeared to lack β-mycolic acid-containing components, II, IV and V. These components were also missing from the TMS-P3 of saprophytic *M. phlei*. Promé *et al.* (18) reported that TMS-cord factor of *M. phlei* contains three components consisting of combinations of "α-phlei-mycolic acid" and "γ-phlei-mycolic acid". Our chromatogram apparently corresponded with theirs, but with the addition of several unidentified minor components.

In light of the significant host-reactive and immunologic properties of trehalose mycolates (1, 21-33), which include the enhancement of tumor immune responses (12, 34-37), it is important to have more precise knowledge of structural features which may be essential for eliciting these properties. Synthetic trehalose diesters of α- or γ-mycolic acids equalled the effectiveness of natural P3 fractions in vaccines that regressed line-10 tumors in strain-2 guinea pigs, indicating that variations in mycolic acid constituency may not appreciably alter activity in this system (12,19,38). However, the presence or absence of a given component might be of considerable significance in other regards, such as pathogenesis or virulence. Continued interest in the potent

immunological properties of trehalose mycolates re-emphasizes the importance of obtaining and testing properly characterized homogeneous compounds. Attempts will be made to upgrade the present analytical techniques to obtain sufficient material to permit detailed structural and biological evaluation of these individual fractions.

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