JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 1976, p. 81-85

Vol. 3, No. 2
Printed in U.S.A.

Gas Chromatographic Characterization of Mycobacteria: Analysis of Fatty Acids and Trifluoroacetylated Whole-Cell Methanolysates

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Received for publication 23 September 1975

Mycobacterium avium, M. bovis strain Bacillus Calmette-Guérin (BCG), *M. kansasii,* and *M. tuberculosis* were studied by gas-liquid chromatography. Methylated fatty acids of the mycobacterial lipids and trifluoroacetyl (TFA) derivatives of whole-cell methanolysates were analyzed. Both the fatty acid and the TFA chromatograms showed reproducible differences between the various mycobacteria studied. Chromatograms from different strains of one and the same species showed negligible differences. Fatty acid methyl esters and TFA methyl glycosides are probably the main constituents of the TFA chromatograms. TFA derivatives are easily prepared and the method provides a potential tool for species identification of mycobacteria.

The application of traditional methods for the identification of mycobacteria is a rather intricate and time-consuming task (8). There is consequently a need for more rapid methods.

In recent years, gas-liquid chromatography (GLC) has been used as a tool for the identification of mycobacteria. Fatty acids of mycobacteria, converted to suitable derivatives, have been analyzed by GLC (12, 19). Analysis of pellets of lyophilized mycobacterial cells by pyrolysis-GLC has also been done (14-17).

GLC analysis of trimethylsilated (TMS) or trifluoroacetylated (TFA) methanolysates of whole cells has been reported to be useful for the identification of bacteria (4, 5, 7, 9). After mild fragmentation of lyophilized cells with methanolic hydrogen chloride, nonvolatile compounds are converted to volatile TMS or TFA derivatives. This method has the advantage that derivatives of bacterial fatty acids and carbohydrates can be detected in one and the same chromatogram (9).

In the present investigation fatty acids and TFA derivatives of whole-cell methanolysates of some species of mycobacteria have been studied by GLC. The study was made to evaluate the possibility of using GLC analysis of such preparations as a tool for the differentiation of mycobacteria.

MATERIALS AND METHODS

Microorganisms. One strain of *Mycobacterium avium* (NCTC 8551) was used. One strain of *M. kansasii* was obtained from J. Stanfield, Middlesex Hospital, London. *M. tuberculosis,* strain $H_{37}R_V$, came from Statens Serum Institute, Copenhagen. Four strains each of *M. tuberculosis* and *M. bovis* (strain Bacillus Calmette-Guérin [BCG]) and two strains of *M. kansasii* were used. All 10 strains were isolated from clinical specimens sent to our laboratory. The BCG strains were isolated from recently vaccinated children; two of the strains were isolated from material collected from suppurating inguinal lymph nodes, whereas the other two were recovered from aspirates of osteomyelitic lesions.

Culture technique. The bacteria were cultured on Löwenstein-Jensen medium for 10 days at 37 C. Colonies from these cultures were inoculated into 200 ml of Proskauer-Beck medium (Difco) and incubated for 30 days in an atmosphere of 8% carbon dioxide. After autoclaving, the broth medium was centrifuged at 4,000 rpm for 20 min. The deposit was washed twice in distilled water, lyophilized, and stored in screw-capped glass tubes under nitrogen at −20 C. As controls, uninoculated culture media were incubated and analyzed under the same conditions as the inoculated media. All tests were made in duplicate. The strain under study was cultured on two slants of Löwenstein-Jensen medium, and from each of the two slants bacteria were inoculated into different batches of Proskauer-Beck medium.

Chemicals. All chemicals, specified in the text, were reagent grade and used without further purification. Ethyl acetate was stored over a molecular sieve.

Methanolysis. Lyophilized cells, in portions of 4 to 6 mg, were stored overnight in a vacuum desiccator containing phosphorous pentoxide. A 1-ml amount of dry 0.5 N methanolic HCl was added, and the methanolysis was allowed to proceed under nitrogen in 12-ml glass tubes for 15 h at 80 C. A piece of Teflon tape was placed under the screw caps to

prevent any extraction from the rubber seals. To evaluate the effect of the HCl concentration, 1 N instead of 0.5 N HCl was used in some experiments.

Preparation of bacterial fatty acids. The fatty acid methyl esters, obtained from the methanolysis, were extracted twice with 5 ml of *n*-hexane. After washing with distilled water, the hexane phase was concentrated to dryness with a stream of dry nitrogen at 40 C. A 100-μl amount of *n*-hexane was added, and 1 to 2 μl of the mixture was injected into the gas chromatograph.

Preparation of TFA derivatives. After methanolysis, the heterogeneous mixture was centrifuged at 5,000 rpm for 5 min and the supernatant was concentrated to dryness with a stream of dry nitrogen at 40 C. Ethyl acetate (50 μl) and 100 μl of trifluoroacetic acid anhydride were added. In some experiments, ethyl acetate was replaced by acetonitrile or pyridine. After 5 min at 80 C, followed by 20 to 30 min at room temperature, 1 to 2 μl of the solution was injected into the gas chromatograph. To evaluate the time required for a complete TFA derivatization, the reaction period was varied from 1 to 15 min at 80 C and from 1 min to 3 days at room temperature.

GLC equipment and test conditions. A Hewlett-Packard gas chromatograph (model 5750) equipped with dual flame-ionization detectors and 6-foot (ca. 182.8-cm; 4 mm ID) glass columns, was used in all experiments. The nitrogen carrier-gas flow was 50 ml/min, and the injector and detector temperature was 310 C. A 3% OV-101 stationary phase on Chromosorb G AW DMCS, 100 to 120 mesh (WGA, London), was regularly used. The column temperature was programmed from 100 to 300 C, with a temperature increase of 6°/min. In some experiments, a 3.8% UCCW stationary phase on Chromosorb W AW DMCS 80 to 100 mesh (WGA, London), was used.

RESULTS

TFA analysis. TFA chromatograms of each of the four mycobacterial species studied, eluting from a 3% OV-101 column, are shown in Fig. 1 through 4. Each species produced similar, but not identical, chromatographic patterns. Seven of the most prominent peaks, labeled a to g in the figures, had approximate retention times of 5, 8, 8.5, 20, 22, 22.5, and 23 min, respectively. Reproducible quantitative differences with regard to the relative peak heights were found for the different species studied. The chromatogram of the strain of *M. avium* studied was characterized by the small preceding shoulder of peak d, the double peak g, and increasing heights of peaks e, f, and g in that order. The strains of *M. kansasii* could be distinguished from that of *M. avium* by the large peak c and decreasing heights of peaks e, f, and g. The largest peak found in the chromatograms produced by the strains of both *M. tuberculosis* and *M. bovis* strain BCG was peak a, whereas peak f was always smaller

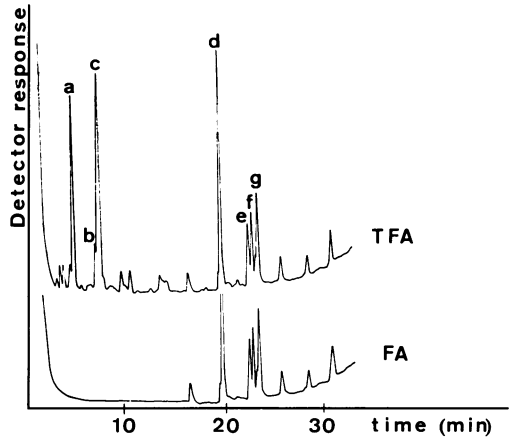

FIG. 1. *TFA and fatty acid (FA) chromatographic elution profiles of M. avium.*

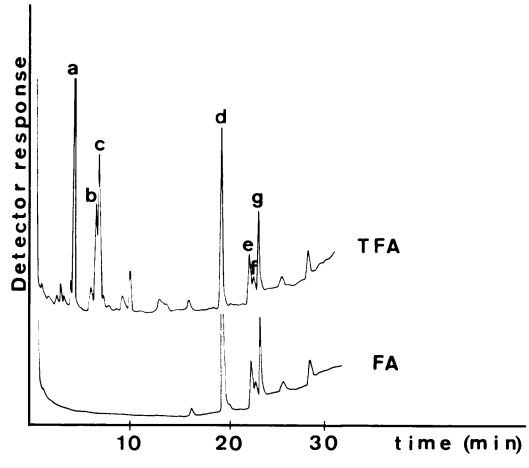

FIG. 2. *TFA and fatty acid (FA) chromatographic elution profiles of M. bovis strain BCG.*

than peaks e and g. The strains of *M. tuberculosis* and *M. bovis* strain BCG produced very similar chromatographic patterns. Only one difference, though reproducible, among peaks a to g was found. In the chromatograms of *M. tuberculosis*, the relative height of peak b compared with peak c was approximately one-third, whereas the corresponding height ratio in the chromatograms of *M. bovis* strain BCG was approximately two-thirds.

Reproducibility of TFA chromatograms. The two chromatograms obtained from material prepared from the two slants of Löwenstein-Jensen medium inoculated with each strain tested showed negligible differences. Multiple analysis of specimens of one and the same strain from repeated cultures always yielded superimposable chromatograms. The

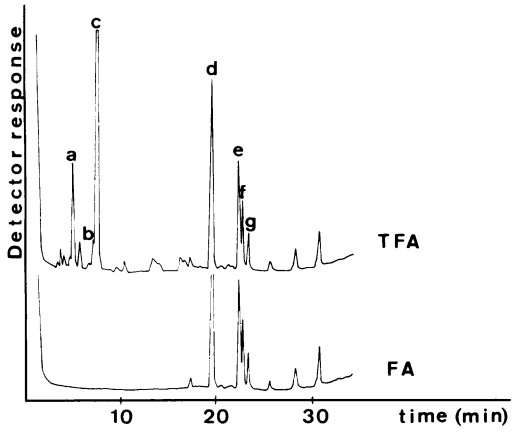

FIG. 3. *TFA and fatty acid (FA) chromatographic elution profiles of M. kansasii.*

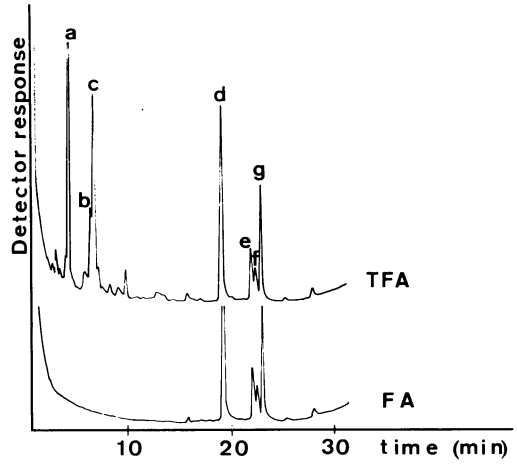

FIG. 4. *TFA and fatty acid (FA) chromatographic elution profiles of M. tuberculosis.*

reproducibility was also estimated by analysis of different strains belonging to the same species. Strains of *M. tuberculosis* isolated from four patients produced chromatograms that did not differ with respect to peaks a to g. Chromatograms of *M. tuberculosis* strain $H_{37}R_v$ and the four strains of this species isolated from clinical specimens sent to our laboratory were superimposable. This was also the case for the four strains of *M. bovis* strain BCG and for the reference strain and the two strains of *M. kansasii* isolated from the corresponding number of patients.

Each time a mycobacterial culture was analyzed, uninoculated culture medium was also chromatographed. No peaks disturbing the fatty acid or TFA chromatographic patterns were found. In the preparation of derivatives, the use of 1 N HCl instead of 0.5 N HCl in the methanolysis process did not affect the chromatographic elution profiles, thus indicating that the lower concentration of HCl was sufficient for a complete reaction. Various solvents for the TFA reaction were compared. Ethyl acetate was found to give a smaller peak tailing than acetonitrile and pyridine and was consequently preferred.

Fatty acid analysis. The chromatographic patterns of the mycobacterial fatty acids are also apparent from Fig. 1 through 4. The same gas chromatographic conditions were used as for the TFA analysis. As can be seen in the figures, the latter parts of the fatty acid and TFA chromatograms are identical. These two types of chromatograms were obtained from different cultures of one and the same strain. Analysis of duplicate samples and repeated analysis of one and the same strain gave highly reproducible fatty acid chromatograms. The use of 1 N instead of 0.5 N HCl did not affect the fatty acid chromatographic patterns.

TFA reaction conditions. A reaction time of 5 min at 80 C, followed by 20 to 30 min at room temperature, was found to provide complete TFA derivatization. These chromatograms were identical to those obtained by analysis of TFA mixtures that had been stored for up to 3 days at room temperature. On the other hand, a mere 1-min reaction at 80 C, followed by direct injection into the gas chromatograph, yielded very small a, b, and c peaks.

GLC column variations. Various silicone stationary phases can be used for the separation of fatty acid methyl esters and TFA-derivatized methyl glycosides. The OV-101 and UCCW phases tested were both useful for the GLC analysis made. However, the OV-101 stationary phase was preferred since considerable bleeding was observed in the last part of the chromatogram when the UCCW phase was used.

DISCUSSION

Lucchesi et al. (12) and Thoen et al. (19) studied the fatty acids of some mycobacterial strains and found reproducible differences between the various species studied. The chromatographic elution profiles obtained were very similar to our fatty acid chromatograms.

Pyrolysis-GLC has been used by Reiner et al. (14–17), and the pyrochromatograms of the various mycobacterial species showed very small, though reproducible, differences. Pyrolysis-GLC has the disadvantage in that it produces peaks that represent unspecified fragments of pyrolyzed material. The method is usually difficult to standardize and interlaboratory repro-

ducibility is generally poor (6, 11). In contrast to pyrolysis-GLC, products from the TFA derivatization method, as used in this study, can be analyzed with a conventional gas chromatograph.

GLC analysis of TMS and TFA derivatives of whole-cell methanolysates has been used for the characterization of *Neisseria* (9). TFA chromatograms of such bacteria were found to be more reproducible and detailed than corresponding TMS chromatograms. Additional evidence on the reproducibility of the TFA derivatization method was obtained in the present study of mycobacteria.

Acetonitrile, ethyl acetate, and pyridine have all been used as solvents for TFA derivatization of monosaccharides (2, 7, 9, 18, 20). We found ethyl acetate to be just as useful as acetonitrile and pyridine, and, since the latter two solvents are poisons, ethyl acetate is preferred. Furthermore, ethyl acetate was also found to produce the smallest peak tailing.

Zanetta et al. (20) studied the release of methylated monosaccharides and fatty acids from various organic materials by acid-catalyzed methanolysis. Complete reaction was attained after approximately 15 h at 80 C in 0.5 N methanolic hydrogen chloride. No peptide bindings are affected by using this mild fragmentation (9). This was confirmed in our studies by the use of a higher acid concentration in some experiments, which did not affect the fatty acid or TFA chromatograms. In our study, we used autoclaved mycobacteria to eliminate any risk of laboratory infection. Thoen et al. (19) have shown that autoclaving of mycobacteria does not affect the GLC fatty acid profile.

No attempt at chemical identification of the GLC peaks was made in the present study. However, the fatty acid and TFA chromatograms were practically identical between approximately 15- and 30-min retention times, indicating that this part of the TFA chromatogram may represent bacterial fatty acids. Analysis of TFA-derivatized methylated arabinose and galactose gave the same retention times as peaks a and c, respectively. This supports the assumption that arabinose and galactose are the main monosaccharides released by hydrolysis of the mycobacteria studied. Other workers, using other methods, have also found these monosaccharides to be the predominating carbohydrates in mycobacterial cell walls (1, 3, 10, 13).

The main volatile products formed by methanolysis of lyophilized cells under anhydrous conditions, followed by the addition of a TMS or TFA reagent, are assumed to be fatty acid methyl esters and TMS or TFA derivatives of monosaccharides (9). TFA derivatives of various carbohydrates are more volatile and heat stable than corresponding TMS derivatives and are consequently preferred for GLC analysis (2).

The present work focuses particularly on the techniques for the TFA derivatization of mycobacterial whole-cell methanolysates. The result of our study seems to indicate that GLC analysis of such derivatives could be of use for the characterization of the mycobacterial species studied. Chromatograms obtained by the TFA derivatization method provided more extensive information than chromatograms of the mycobacterial fatty acids only. A more extensive survey of other known mycobacterial species is in progress, as is a study on the possibility of analyzing mycobacteria harvested directly from Löwenstein-Jensen medium.

ACKNOWLEDGMENT

This study was supported by the Swedish National Association Against Heart and Chest Diseases.

LITERATURE CITED

1. **Acharya, P. V. N., and D. S. Goldman.** 1970. Chemical composition of the cell wall of the $H_{37}R_a$ strain of *Mycobacterium tuberculosis*. J. Bacteriol. 102:733-739.
2. **Ando, S., and T. Yamakawa.** 1971. Application of trifluoroacetyl derivatives to sugar and lipid chemistry. I. Gas chromatographic analysis of common constituents of glycolipids. J. Biochem. 70:335-340.
3. **Azuma, I., A. Ohuchida, T. Taniyama, Y. Yamamura, K. Shoji, M. Hori, Y. Tanaka, and E. Ribi.** 1974. Mycolic acids of *Mycobacterium rhodochrous* and *Nocardia corallina*. Biken J. 17:1-9.
4. **Farshtchi, D., and C. W. Moss.** 1969. Characterization of bacteria by gas chromatography: comparison of trimethylsilyl derivatives of whole-cell hydrolysates. Appl. Microbiol. 17:262-267.
5. **Farshy, D. C., M. L. Thomas, and C. W. Moss.** 1970. Characterization of treponemes by gas chromatography. Br. J. Vener. Dis. 46:441-444.
6. **Fontanges, R., G. Blandenet, and R. Queignec.** 1967. Difficultés d'application de la chromatographie en phase gazeuse a l'identification des bactéries. Ann. Inst. Pasteur Paris 112:10-23.
7. **Frøholm, L. O., E. Jantzen, R. Hytta, and K. Bøvre.** 1972. Gas chromatography of bacterial whole cell methanolysates. II. A taxonomic evaluation of the method for species of *Moraxella*. Acta Pathol. Microbiol. Scand. Sect. B 80:672-682.
8. **Iwainsky, H., and W. Käppler.** 1974. Mykobakterien. Biochemie und biochemische Differenzierung, p. 141-219. Johann Ambrosius Barth,Leipzig.
9. **Jantzen, E., L. O. Frøholm, R. Hytta, and K. Bøvre.** 1972. Gas chromatography of bacterial whole cell methanolysates. I. The usefulness of trimethylsilyl- and trifluoroacetyl derivatives for strain and species characterization. Acta Pathol. Microbiol. Scand. Sect. B 80:660-671.
10. **Kanetsuna, F.** 1968. Chemical analysis of mycobacterial cell walls. Biochim. Biophys. Acta 158:130-143.
11. **Levy, R. L.** 1967. Trends and advances in design of

pyrolysis units for gas chromatography. J. Gas Chromatogr. 5:107-113.
12. **Lucchesi, M., C. Cattaneo, and G. C. deRitis.** 1967. The chromatographic separation of fatty acids (C11-C20) from mycobacteria. Bull. Int. Union Tuberc. 39:65-70.
13. **Misaki, A., and S. Yukawa.** 1966. Studies of cell walls of mycobacteria. II. Constitution of polysaccharides from BCG cell walls. J. Biochem. 59:511-520.
14. **Reiner, E.** 1965. Identification of bacterial strains by pyrolysis-gas-liquid chromatography. Nature (London) 206:1272-1274.
15. **Reiner, E., R. E. Beam, and G. P. Kubica.** 1969. Pyrolysis-gas-liquid chromatography studies for the classification of mycobacteria. Am. Rev. Respir. Dis. 99:750-759.
16. **Reiner, E., J. J. Hicks, R. E. Beam, and H. L. David.** 1971. Recent studies on mycobacterial differentiation by means of pyrolysis-gas-liquid chromatography. Am. Rev. Respir. Dis. 104:656-660.
17. **Reiner, E., and G. P. Kubica.** 1969. Predictive value of pyrolysis-gas-liquid chromatography in the differentiation of mycobacteria. Am. Rev. Respir. Dis. 99:42-49.
18. **Shapira, J.** 1969. Identification of sugars as their trifluoroacetyl polyol derivatives. Nature (London) 222:792-793.
19. **Thoen, C. O., A. G. Karlson, and R. D. Ellefson.** 1971. Comparison by gas-liquid chromatography of the fatty acids of *Mycobacterium avium* and some other nonphotochromogenic mycobacteria. Appl. Microbiol. 22:560-563.
20. **Zanetta, J. P., W. C. Breckenridge, and G. Vincendon.** 1972. Analysis of monosaccharides by gas-liquid chromatography of the o-methyl glycosides as trifluoroacetate derivatives. Application to glycoproteins and glycolipids. J. Chromatogr. 69:291-304.