

Use of Gas Chromatography to Differentiate *Mycobacterium leprae* from Cultivable Armadillo-derived Mycobacteria, *M. avium/intracellulare*, and *M. lepraemurium* by Analysis of Secondary Alcohols¹

Lennart Larsson, Philip Draper, and Françoise Portaels²

The experimentally infected, nine-banded armadillo is at present the major source of *Mycobacterium leprae* for microbiological study⁽⁹⁾. A purified suspension from this source is also a candidate antileprosy vaccine. Consequently, the report⁽¹⁷⁾ that a substantial proportion of *M. leprae*-containing armadillo tissues also contained other mycobacterial species was disturbing. One particular series of strains, which required special growth conditions, appeared sometimes to be present in substantial amounts^(17, 18). The possibility arose that suspensions prepared for vaccine studies were significantly contaminated by armadillo-derived mycobacteria (ADM). Such contamination would confuse microbiological and immunological studies on the suspensions, and might also give rise to problems of safety.

Fortunately, chemical differences exist between *M. leprae* and ADM. For example, "dicarboxylic" mycolic acids have been found in strains of ADM⁽¹⁵⁾ but do not seem to be present in *M. leprae*^(2, 5, 7, 8, 21). Thin-layer chromatography (TLC) has been used to distinguish *M. leprae* in human tissues from various mycobacteria cultivated from human skin biopsies, based on the presence of these "dicarboxylic" mycolic acids in the cultivable strains⁽²¹⁾.

The "dicarboxylic" mycolic acids contain an internal ester function; hydrolysis of the parent compound yields a dicarboxylic acid and an alcohol. 2-Octadecanol and 2-eico-

sanol from this source have been detected in certain mycobacterial hydrolysates using gas chromatography (GC) and found to constitute important markers in species differentiation⁽³⁾. GC is a sensitive method for detection and quantification of such long-chain alcohols, analyzed either underivatized or, e.g., as trifluoroacetyl ethers^(1, 12).

The present investigation was carried out in order to study the distribution of 2-octadecanol and 2-eicosanol in *M. leprae* and ADM and for comparison also in *M. avium/intracellulare* and *M. lepraemurium* grown *in vivo*. The potential use of these secondary alcohols for measuring a possible population of ADM in preparations of *M. leprae* is discussed.

MATERIALS AND METHODS

Cultivated organisms. Four strains each of ADM and *M. avium/intracellulare* were analyzed (The Table). The ADM were isolated from livers of armadillos experimentally infected with *M. leprae* and cultivated in Dubos or in Middlebrook 7 H9 medium. *M. avium/intracellulare*, isolated from clinical specimens at the Bacteriological Department, University Hospital, Lund, Sweden, and *M. avium* (NCTC 8551) were cultivated in Proskauer-Beck liquid medium⁽¹²⁾. After checking for purity, the bacteria were killed by autoclaving or with formalin (1% v/v), harvested by centrifugation, washed with distilled water, and lyophilized before being subjected to hydrolysis and GC analysis.

Organisms from tissues. All of the *M. leprae* suspensions were obtained from tissues (liver, spleen or lymph node) of experimentally infected, nine-banded armadillos using the so-called Protocol 1/79⁽²⁰⁾. Twenty of the suspensions were samples of material prepared by Wellcome Laboratories (U.K.)

¹ Received for publication on 2 January 1985; accepted for publication in revised form on 2 April 1985.

² L. Larsson, Ph.D., Head, Department of Medical Microbiology, University of Lund, S-223 62 Lund, Sweden; P. Draper, D.Phil., National Institute for Medical Research, Mill Hill, London NW7 1AA, England; F. Portaels, Dr.Sc., Institute for Tropical Medicine, B-2000 Antwerp, Belgium.

THE TABLE. List of the mycobacterial strains studied.

Bacterium	Designation of strain/preparation	Cultivation
<i>M. avium</i>	NCTC 8551	Proskauer-Beck medium
<i>M. avium/intracellulare</i>	R 7	Proskauer-Beck medium
<i>M. avium/intracellulare</i>	R 13	Proskauer-Beck medium
<i>M. avium/intracellulare</i>	R 53	Proskauer-Beck medium
<i>M. lepraemurium</i>	"Douglas" Mellanby	Mouse spleen
<i>M. lepraemurium</i>	"Douglas" Closs	Mouse spleen
<i>M. lepraemurium</i>	"Douglas" Mellanby	Mouse liver
<i>M. lepraemurium</i>	"Douglas" Closs	Mouse liver
<i>M. lepraemurium</i>	"Douglas" W44	Mouse tissues ^d
<i>M. lepraemurium</i>	Hawaii 558	Mouse tissues
<i>M. lepraemurium</i>	Cat 694	Mouse tissues
<i>M. lepraemurium</i>	Rat 693	Mouse tissues
<i>M. lepraemurium</i>	Wong 696	Mouse tissues
<i>M. leprae</i>	"Wellcome" no. 3, 27, 30	Armadillo liver no. R 88
<i>M. leprae</i>	"Wellcome" 17, 18	Armadillo liver 2359
<i>M. leprae</i>	"Wellcome" 23, 31, 33	Armadillo liver 2443
<i>M. leprae</i>	"Wellcome" 29	Armadillo liver 2318
<i>M. leprae</i>	"Wellcome" 22, 34, 35, 36	Armadillo liver 2302
<i>M. leprae</i>	"Wellcome" 37, 38, 39	Armadillo liver 2519
<i>M. leprae</i>	"Wellcome" 44, 50, 51	Armadillo liver 2548/218
<i>M. leprae</i>	"Wellcome" 48	Armadillo liver 12
<i>M. leprae</i>	PDPE	Armadillo liver 2236
<i>M. leprae</i>	I2 BZ	Armadillo spleen 12
<i>M. leprae</i>	CD 20	Armadillo tissues ^d (several animals)
ADM	8346 ^a	Dubos medium
ADM	8507 ^b	Middlebrook 7H9 medium
ADM	8634 ^c	Middlebrook 7H9 medium
ADM	8637 ^a	Middlebrook 7H9 medium

^a Isolated from armadillo liver AJ (Dr. Y. Robin, Cayenne).

^b Armadillo liver no. 2457/10 (Dr. R. J. W. Rees, London).

^c Liver no. 44 (Dr. C. C. Shepard, Atlanta, Georgia, U.S.A.).

^d "Tissues" indicates a mixture of spleen and liver in the case of *M. lepraemurium* and of spleen, liver and lymph node in the case of *M. leprae*.

for the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases for use in vaccine trials. The remaining three suspensions were prepared at the National Institute for Medical Research, London.

Suspensions of *M. lepraemurium* were prepared by the same method from livers and spleens of mice infected with a massive (10^9 organisms) intravenous inoculum. Strain Douglas is commonly used experimentally in Europe, strain Hawaii in the United States and Japan. The sub-strains Mellanby and Closs have different virulence for mice. W44 was a preparation of purified walls of *M. lepraemurium* prepared by the method of Draper (6). Details of the various suspensions are given in The Table.

Hydrolysis and derivatization. The ly-

ophilized cells, in 0.5–1.0 mg portions, were heated in 0.5 ml of 1 N methanolic HCl overnight at 80°C. The suspensions were then centrifuged and the supernatants evaporated to dryness under a stream of dry nitrogen. One hundred μ l of *n*-hexane was added to each sample and, after shaking, the organic phases were transferred to new test tubes. After evaporation, 10–20 μ l of *n*-hexane was added. This extract was then analyzed by GC.

Each of the final preparations was also subjected to trifluoroacetylation and re-analysis in order to confirm the presence or absence of 2-octadecanol and 2-eicosanol. After evaporation to dryness, 50 μ l each of acetonitrile and trifluoroacetic anhydride were added. After heating for 5 min at 80°C, the samples were allowed to cool to room

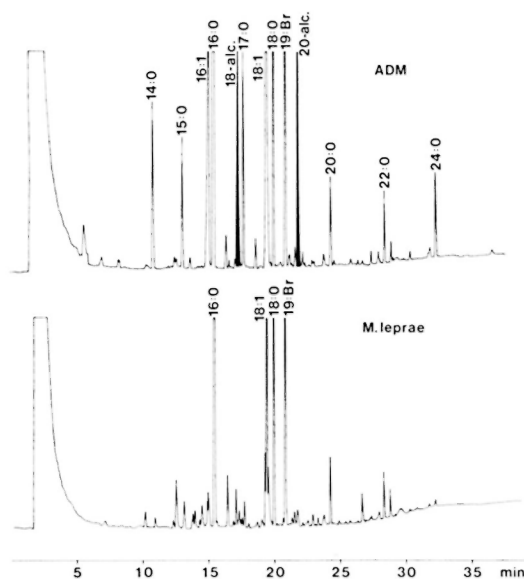


FIG. 1. Chromatograms representing cellular fatty acid methyl esters and secondary alcohols in an armadillo-derived mycobacterial strain (ADM no. 8634) and in *M. leprae* (Wellcome Strain no. 18). The designations of the fatty acid peaks refer to the number of carbon atoms and double bonds; 19:Br is tuberculostearic acid ester. Blackened peaks represent 2-octadecanol (18-alc) and 2-eicosanol (20-alc), respectively. (See text for specifications of cultivation, derivatization and gas chromatographic conditions.)

temperature and evaporated. Ten to 20 μ l of *n*-hexane was added and the samples immediately subjected to GC analysis.

Gas chromatography. A Varian model 2400 gas chromatograph was used. The chromatograph was equipped with a capillary injector and a 25 m fused silica capillary column (i.d. 0.2 mm), coated with SE-54. Sample introduction was performed using splitless injection⁽¹⁴⁾ of approximately 0.5 μ l of each preparation. The temperature of the injector was 280°C and that of the detector 295°C. The temperature of the column was programmed from 160–290°C, with a rate of 4°/min. Nitrogen was used both as carrier and make-up gas at flow rates of 0.9 and 17 ml/min, respectively. The attenuation of the flame ionization detector signal was 16.

RESULTS

Representative chromatograms are shown in Figures 1 and 2. The major fatty acid peaks represented the methyl esters of pal-

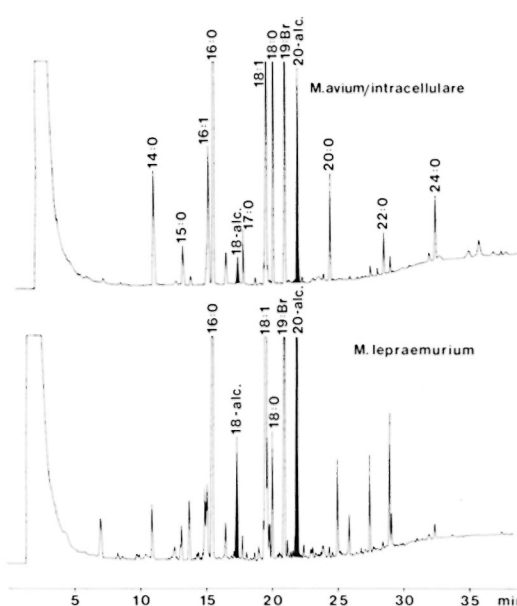


FIG. 2. Chromatograms representing cellular fatty acid methyl esters and secondary alcohols in *M. avium/intracellulare* (Strain no. R7) and *M. lepraemurium* (Mellanby liver). (See legend to Fig. 1 and text for symbols and technical details.)

mitic (C 16:0), octadecenoic (C 18:1), stearic (C 18:0; smallest in *M. lepraemurium*) and tuberculostearic acid (C 19:Br). In addition, saturated fatty acids with 14, 15, 17, 20, 22, and 24 carbon atoms were found. Only small differences (minor variations in the relative peak areas) were observed between the chromatograms within each of the four groups of mycobacteria studied. In all strains except *M. leprae*, 2-octadecanol (C 18-alc) and 2-eicosanol (C 20-alc) were detected. Absence or presence of these secondary alcohols in the strains studied was confirmed by trifluoroacetylation followed by re-analysis of the extracts. The chromatograms representing the *M. leprae* strains were identical before and after trifluoroacetylation, thereby excluding the possibility that any of the small peaks found in positions close to those of 2-octadecanol and 2-eicosanol would actually correspond to these alcohols⁽¹²⁾.

DISCUSSION

It was recently reported that it can be difficult to determine the proportions of cultivable mycobacteria (i.e., not *M. leprae*)

present in *M. leprae*-infected armadillo tissues if cultivation methods only are used⁽¹⁶⁾. For rapid demonstration of ADM contamination, before results from culture studies become available, pyrolysis GC methods for detection of alkenes derived from carboxy-mycolates⁽¹⁹⁾ and use of monoclonal antibodies specific for some ADM strains for *in situ* detection of mycobacterial contaminants⁽¹⁰⁾ have been used. Both methods might be capable of estimating the amount of contaminants present. In the present investigations we found that 2-octadecanol and 2-eicosanol were useful chemical markers of ADM contamination, and that GC was a sensitive method for their detection and quantification.

The two secondary alcohols studied—2-octadecanol and 2-eicosanol—can be released from “dicarboxylic” mycolic acid-containing mycobacteria using either acid methanolysis or alkaline saponification, the same methods as frequently used in the preparation of methyl esters of mycobacterial fatty acids for strain and species characterization by GC analysis. It has been demonstrated, however, that unless certain precautions are taken, namely, removal of excess of reagents and/or free, underivatized fatty acids from the final extract used for GC analysis, the alcohols may be partly or totally adsorbed on the column and thus not registered by the detector⁽¹²⁾. In fact, these alcohols are not included in most published chromatograms of mycobacteria containing the “dicarboxylic” mycolic acids. By contrast, they have been frequently detected in thin-layer chromatographic (TLC) systems. However, compared to GC most TLC methods are considerably less sensitive and are, at best, semi-quantitative.

Our finding of 2-octadecanol and 2-eicosanol both in mycobacteria grown *in vivo* (*M. lepraemurium*) and *in vitro* (ADM, *M. avium/intracellulare*) shows that the absence of these alcohols in *M. leprae* is not related to the fundamental differences between *in vivo* and *in vitro* cultivation. The results support previous findings where identical mycolic acid patterns, including the consistent presence of “dicarboxylic” mycolic acids, were found in *M. lepraemurium* cultivated both *in vivo* and *in vitro*⁽¹¹⁾, while *M. microti* produces the same

pattern of mycolic acids, not including a “dicarboxylic” component, whether grown *in vivo* or *in vitro*⁽⁴⁾.

The absence of “dicarboxylic” mycolic acids from mycobacterial suspensions does not, of course, demonstrate that the organisms are *M. leprae*. Nor does it demonstrate that species of mycobacteria not containing “dicarboxylic” mycolic acids are absent from such suspensions. These studies were undertaken to show that substantial contamination with a particular type of mycobacterium, shown to be present in some of the tissues used for preparing suspensions of *M. leprae*⁽¹⁷⁾, did not occur in the suspensions intended for use in humans. The method described is useful in ruling out contamination by organisms of the so-called MAIS group as well as by *M. lepraemurium*, a difficult-to-grow organism which, as a pathogen of small rodents, has many times been confused with *M. leprae* in attempts to grow the latter in animals. The possibility that a search for long-chain, secondary alcohols should form part of the quality control procedures for suspensions of *M. leprae* should be considered. Further, since armadillo tissues seem likely to be the main source of *M. leprae* for some time to come, it remains important to gain more knowledge of ADM, their occurrence and properties.

Although not demonstrated in the present study, it is obvious that GC can be used not only to detect but also to measure ADM contamination in *M. leprae* suspensions from experimentally infected armadillos. Further, the technique may also be valuable for checking the purity of “*in vitro* cultivated” *M. leprae*. The sensitivity of the GC method might be increased, if necessary, by the use of mass spectrometric or electron capture detection, e.g., of heptafluorobutyl alcohol derivatives. Such detection modes have previously been applied successfully to other mycobacterial constituents, e.g., for determination of trace amounts of tuberculostearic acid in clinical specimens where a thousand-fold increase in sensitivity has been achieved over methods using flame ionization detection⁽¹³⁾.

SUMMARY

Two long-chain secondary alcohols, 2-octadecanol and 2-eicosanol, were dem-

onstrated by gas chromatography in hydrolysates of *Mycobacterium avium/intracellulare*, in cultivable, armadillo-derived mycobacteria, and in *M. lepraemurium* grown *in vivo*, but they were not found in purified suspensions of *M. leprae* isolated from experimentally infected armadillos. Gas chromatographic analysis of these alcohols constitutes a method for rapid detection and quantification of contaminating mycobacteria in preparations of *M. leprae* intended, for example, for vaccine use. The technique may also be of value for critical evaluation of cultures of "in vitro-grown" *M. leprae*.

RESUMEN

Empleando la cromatografía de gases, se identificaron dos alcoholes secundarios de cadena larga (2-octadecanol y 2-eicosanol) en los hidrolizados de (a) *Mycobacterium avium/intracellulare*, (b) de las micobacterias derivadas de armadillo, y (c) de *M. lepraemurium* crecido *in vivo*, pero no se encontraron en las suspensiones purificadas del *M. leprae* aislado de armadillos infectados experimentalmente. El análisis por cromatografía de gases de estos alcoholes constituye un método para la rápida detección y cuantificación de micobacterias que contaminan las preparaciones de *M. leprae* destinadas, por ejemplo, para su uso como vacuna. La técnica puede ser útil también para la evaluación crítica de los cultivos de *M. leprae* "crecidos *in vitro*."

RÉSUMÉ

Deux alcools secondaires de chaîne longue (2-octadecanol et 2-eicosanol) ont été mis en évidence par chromatographie en phase gazeuse d'hydrolysats de *Mycobacterium avium/intracellulare*, de mycobactéries cultivables dérivées de tatous et de *M. lepraemurium* cultivé "in vivo." Ces alcools n'ont pas été détectés dans des préparations purifiées de *M. leprae* provenant de tatous infectés expérimentalement.

L'analyse de ces alcools par chromatographie en phase gazeuse permettrait de détecter rapidement et de manière quantitative, la présence de mycobactéries autres que *M. leprae* dans des préparations de *M. leprae* destinées à la vaccination. Cette méthode pourrait également être appliquée à l'étude d'éventuelles cultures "in vitro" de *M. leprae*.

Acknowledgments. Many of the suspensions of *M. leprae* used were prepared by Wellcome Laboratories for the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. We are grateful to the IMMLEP Steering Committee of the Programme for making the samples available.

Tissues containing *Mycobacterium lepraemurium* were gifts from Dr. J. L. Stanford and Mr. R. Butler.

Grants from the Swedish National Association against Heart and Chest Diseases; the Leprosy Unit, WHO (Geneva); and the Damien Foundation (Brussels) are gratefully acknowledged.

REFERENCES

1. ALVIN, C., LARSSON, L., MAGNUSSON, M., MÅRDH, P.-A., ODHAM, G. and WESTERDAHL, G. Determination of fatty acids and carbohydrate monomers in microorganisms using capillary gas chromatography: Analysis of *Mycobacterium gordonae* and *Mycobacterium scrofulaceum*. J. Gen. Microbiol. **129** (1983) 401-405.
2. DAFFÉ, M., LANEÉLLE, M. A., ASSELINEAU, C., CLAVEL, S., CLEMENT, F. and DAVID, H. L. Étude comparative des lipides de *Mycobacterium leprae* et des autres souches de mycobactéries. Ann. Microbiol. (Paris) **133B** (1982) 49-51.
3. DAFFÉ, M., LANEÉLLE, M. A., ASSELINEAU, C., LÉVY-FRÉBAULT, V. and DAVID, H. Intérêt taxonomique des acides gras des mycobactéries: Proposition d'une méthode d'analyse. Ann. Microbiol. (Paris) **134B** (1983) 241-256.
4. DAVIDSON, L. A., DRAPER, P. and MINNIKIN, D. E. Studies on the mycolic acids from the walls of *Mycobacterium microti*. J. Gen. Microbiol. **128** (1982) 823-828.
5. DRAPER, P. Cell walls of *Mycobacterium leprae*. Int. J. Lepr. **44** (1976) 95-98.
6. DRAPER, P. The walls of *Mycobacterium lepraemurium*: Chemistry and ultrastructure. J. Gen. Microbiol. **69** (1971) 313-324.
7. DRAPER, P., DOBSON, G., MINNIKIN, D. E. and MINNIKIN, S. M. The mycolic acids of *Mycobacterium leprae* harvested from experimentally infected nine-banded armadillos. Ann. Microbiol. (Paris) **133B** (1982) 39-47.
8. ETÉMADI, A. H. and CONVIT, J. Mycolic acids from "non-cultivable" mycobacteria. Infect. Immun. **10** (1974) 235-239.
9. KIRCHHEIMER, W. F. and STORRS, E. E. Attempts to establish the armadillo (*Dasypus novemcinctus* Linn.) as a model for the study of leprosy. 1. Report of lepromatoid leprosy in experimentally infected armadillo. Int. J. Lepr. **39** (1971) 693-702.
10. KOLK, A. H. J., HO, M. L., KLATSER, P. R., EGELTE, T. A. and PORTAELS, F. The use of monoclonal antibodies directed against *Mycobacterium leprae* and armadillo derived mycobacteria. Program and Abstracts of the WHO-ESM-SFM Joint Meeting on *Mycobacterium leprae*, 12-14 June 1984, Institut Pasteur, Paris, p. 67.
11. KUSAKA, T., KOHSAKA, K., FUKUNISHI, Y. and AKIMORI, H. Isolation and identification of mycolic acids in *M. leprae* and *M. lepraemurium*. Int. J. Lepr. **49** (1981) 406-416.
12. LARSSON, L. Acidic methanolysis v. alkaline saponification in gas chromatographic characterization of mycobacteria: Differentiation between *Mycobacterium avium/intracellulare* and *Mycobacterium leprae*.

- bacterium gastris*. Acta Pathol. Microbiol. Immunol. Scand. [B] **91** (1983) 235-239.
13. LARSSON, L., MÅRDH, P.-A., ODHAM, G. and WESTERDAHL, G. Detection of tuberculostearic acid in biological specimens by means of glass capillary gas chromatography/electron and chemical ionization mass spectrometry, utilizing selected ion monitoring. J. Chromatogr. Biomed. Appl. **182** (1980) 402-408.
 14. LARSSON, L. and ODHAM, G. Injection principles in capillary gas chromatographic analysis of bacterial fatty acids. J. Microbiol. Meth. **3** (1984) 77-82.
 15. PORTAELS, F., DAFFÉ, M., LANEËLLE, M. A. and ASSELINEAU, C. Étude de la composition lipidique de mycobactéries cultivables isolées de foies de tatous infectés par *Mycobacterium leprae*. Ann. Microbiol. (Paris) **135A** (1984) 457-465.
 16. PORTAELS, F., FRANCKEN, A. and DE RIDDER, K. Culture de mycobactériés à partir de tissus infectés expérimentalement par *M. leprae*. Program and Abstracts of the WHO-ESM-SFM Joint Meeting on *Mycobacterium leprae*, 12-14 June 1984, Institut Pasteur, Paris, p. 53.
 17. PORTAELS, F., FRANCKEN, A. and PATTYN, S. R. Bacteriological studies of armadillo livers infected with *Mycobacterium leprae*. Ann. Soc. Belg. Med. Trop. **62** (1982) 233-245.
 18. PORTAELS, F. and PATTYN, S. R. Isolation of fastidiously growing mycobacteria from armadillo livers infected with *Mycobacterium leprae*. Int. J. Lepr. **50** (1982) 370-374.
 19. WIETEN, G., BOON, J. J., GROOTHUIS, D. G., DRAPER, P. and PORTAELS, F. Characterization and screening of *M. leprae* and other mycobacteria by pyrolysis mass spectrometry (Py-MS) and pyrolysis gas chromatography (Py-GC). Program and Abstracts of the WHO-ESM-SFM Joint Meeting on *Mycobacterium leprae*, 12-14 June 1984, Institut Pasteur, Paris, p. 12.
 20. WORLD HEALTH ORGANIZATION. UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Report of the Fifth Meeting of the Scientific Working Group on the Immunology of Leprosy (IMMLEP). TDR/IMMLEP-SWG Annex 4, 123 (5)/80.3. Geneva, 24-26 June 1980.
 21. YOUNG, D. B. Identification of *Mycobacterium leprae*: Use of wallbound mycolic acids. J. Gen. Microbiol. **121** (1980) 249-253.