Use of Gas Chromatography to Differentiate *Mycobacterium leprae* from Cultivable Armadillo-derived Mycobacteria, *M. avium/intracellulare,* and *M. lepraemurium* by Analysis of Secondary Alcohols[1]

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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-eicosanol 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 trifluoracetyl 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.)

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THE TABLE. *List of the mycobacterial strains studied.*

Bacterium	Designation of strain/preparation	Cultivation
M. avium	NCTC 8551	Proskauer-Beck medium
M. avium/intracellulare	R 7	Proskauer-Beck medium
M. avium/intracellulare	R 13	Proskauer-Beck medium
M. avium/intracellulare	R 53	Proskauer-Beck medium
M. lepraemurium	"Douglas" Mellanby	Mouse spleen
M. lepraemurium	"Douglas" Closs	Mouse spleen
M. lepraemurium	"Douglas" Mellanby	Mouse liver
M. lepraemurium	"Douglas" Closs	Mouse liver
M. lepraemurium	"Douglas" W44	Mouse tissues[d]
M. lepraemurium	Hawaii 558	Mouse tissues
M. lepraemurium	Cat 694	Mouse tissues
M. lepraemurium	Rat 693	Mouse tissues
M. lepraemurium	Wong 696	Mouse tissues
M. leprae	"Wellcome" no. 3, 27, 30	Armadillo liver no. R 88
M. leprae	"Wellcome" 17, 18	Armadillo liver 2359
M. leprae	"Wellcome" 23, 31, 33	Armadillo liver 2443
M. leprae	"Wellcome" 29	Armadillo liver 2318
M. leprae	"Wellcome" 22, 34, 35, 36	Armadillo liver 2302
M. leprae	"Wellcome" 37, 38, 39	Armadillo liver 2519
M. leprae	"Wellcome" 44, 50, 51	Armadillo liver 2548/218
M. leprae	"Wellcome" 48	Armadillo liver I2
M. leprae	PDPE	Armadillo liver 2236
M. leprae	I2 BZ	Armadillo spleen I2
M. leprae	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 lyophilized 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 μl of *n*-hexane was added to each sample and, after shaking, the organic phases were transferred to new test tubes. After evaporation, 10-20 μl of *n*-hexane was added. This extract was then analyzed by GC.

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

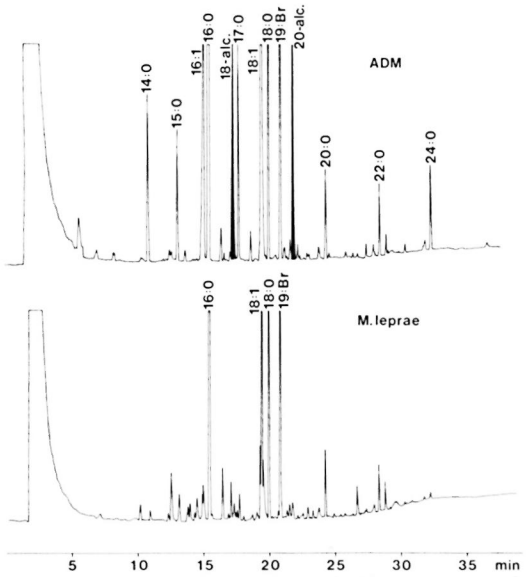

FIG. 1. Chromatograms representing cellular fatty acid methyl esters and secondary alcohols in an armadillo-derived mycobacterial strain (ADM no. 8634) and in *M. leprae* (Wellcome Strain no. 18). The designations of the fatty acid peaks refer to the number of carbon atoms and double bonds; 19:Br is 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.)

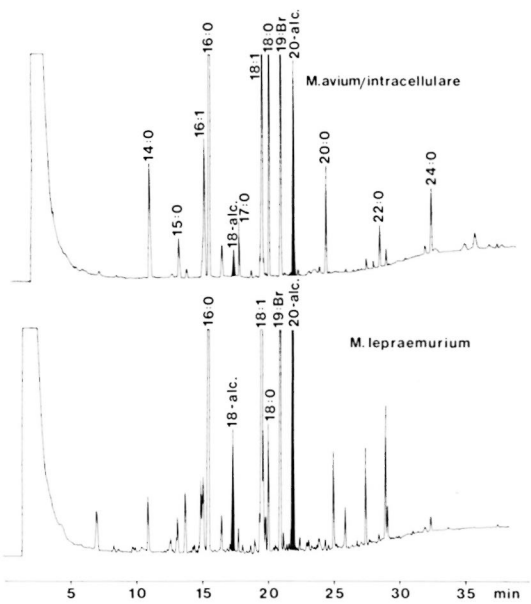

FIG. 2. Chromatograms representing cellular fatty acid methyl esters and secondary alcohols in *M. avium/intracellulare* (Strain no. R7) and *M. lepraemurium* (Mellanby liver). (See legend to Fig. 1 and text for symbols and technical details.)

temperature and evaporated. Ten to 20 μl 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 μl 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 palmitic (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 trifluoracetylation 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 heptafluorobutyryl 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 chaine 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 detectés dans des préparations purifiées de *M. leprae* provenant de tatous infectés experimentalement.

L'analyse de ces alcools par chromatographie en phase gazeuse permettrait de détecter rapidement et de manière quantitative, la presence 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.*

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