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Analysis of cellular fatty acids and proteins by capillary gas chromatography and sodium dodecyl sulphate polyacrylamide gel electrophoresis to differentiate *Mycobacterium avium, Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* (MAIS) complex species

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ABSTRACT

Infections due to atypical mycobacteria have increased during the past 30 years. Species of *Mycobacterium avium, Mycobacterium intracellulare* and *Mycobacterium scrofulaceum* are among the most common non-tuberculous mycobacteria isolated from patients with AIDS or immunosuppressed. These three organisms are taxonomically closely related and identification, according to cultural characteristics and biochemical tests, is not always evident, so some of these related strains are grouped in a "MAIS" complex. Analysis of cellular constituents is an aid to identification. Gas chromatography was used to study mycolic acids and a secondary alcohol was found which is a discriminating constituent between *M. scrofulaceum* and the other two species The lipidic analysis was not able to separate *M. avium* and *M. intracellulare*, so cell proteins were considered Sodium dodecyl sulphate polyacrylamide gel electrophoresis of proteins reflects genetic relatedness between strains; the different patterns obtained from these three species are described and it is shown that this method is very useful in classification and epidemiology

INTRODUCTION

A number of mycobacterial species other than *Mycobacterium tuberculosis* are commonly found in a saprophytic state in the environment Some of these atyp-

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ical mycobacteria can develop in man different syndromes which may mimic infection due to *M. tuberculosis* [1] and may cause invasive disease. Infections due to atypical mycobacteria have been increasingly recognized during the past 30 years. Species of *M. avium*, *M. intracellulare* and *M. scrofulaceum* (MAIS complex) are among the most common non-tuberculous mycobacteria isolated from patients [2]. Immunosuppression, from disease or drugs, is a predisposing factor for the development of mycobacteriosis; in particular disseminated disease is frequently encountered in patients with acquired immunodeficiency syndrome (AIDS) [3]. In these patients, *M. avium* complex species are the most frequent of the atypical mycobacteria isolated from various sites including blood [4–7]. Epidemia of AIDS has increased interest in the MAIS group species.

Most of the non-tuberculous mycobacteria are highly resistant to conventional antimycobacterial drugs [8,9] and identification is necessary to start appropriate therapy. Mycobacteria are classified into four groups on the basis of cultural characteristics and pigmentation [10] and differential identification is based on biochemical reactions [11]. In many cases, the time required to perform biochemical tests is protracted by the low growth rate of mycobacteria, and identification can take up to several weeks for a complete diagnosis. Further, the three organisms, *M. avium*, *M. intracellulare* and *M. scrofulaceum*, are closely related taxonomically. *M. scrofulaceum* differs from *M. avium* and *M. intracellulare* by the presence of a pigment and by the urease reaction; *M. avium* and *M. intracellulare* have a very close relationship and only the rates of rare biochemical characteristics differ. These features are not consistent. *M. scrofulaceum* should be considered as a pigmented form of *M. avium*: some cultures of *M. avium* and *M. intracellulare* are often pigmented. Hence these three species are grouped in a "MAIS complex", a designation proposed to include the strains whose biochemical characteristics are not clearly related to a well defined species [12]. *M. avium* complex and *M. scrofulaceum* coexist in the same group. Identification to the level of species can usually be achieved by biochemical tests. Often, additional analysis may be necessary to lead to a suitable distinction or to have a more rapid diagnosis. Earlier detection of disseminated infection and more rapid identification of mycobacteria will yield better clinical results in the future.

Epidemiological studies need convenient identification and require more elaborate procedures [13–16]; these procedures are based on the analysis of the cellular constituents. Fatty acid analysis appears to be a particularly suitable method for the identification of mycobacteria [17–19]. We have used a gas chromatographic (GC) technique to find a specific marker which differentiates *M. scrofulaceum* from the other two species.

In addition, we report the fractionation and separation of cell proteins to obtain discriminated patterns. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of proteins reflects genetic relatedness between strains and is a useful tool in classification and in epidemiological studies [20,21].

EXPERIMENTAL

Bacterial strains

The following strains were included in the study: *M. avium*, one reference strain ATCC 14031 0005 and one type strain obtained from the laboratory of the Pitié Salpétrière Hospital (Paris, France) (courtesy of Professor J. Grosset); *M. intracellulare*, one reference strain ATCC 14031 0001 and three type strains from Professor J. Grosset; and *M. scrofulaceum*, one reference strain ATCC 14022 0001 and two type strains from Professor J. Grosset. Thirty-one strains were isolated from clinical specimens at the Centre Hospitalier Régional (CHR, Bordeaux, France); twenty-five were *M. avium/M. intracellulare* and six were *M. scrofulaceum*. The strains isolated from biological samples were identified according to standard criteria.

To perform the two techniques, bacteria were cultivated on Löwenstein–Jensen media until growth was sufficient.

Extraction of fatty acids

Details of the techniques were given in a previous paper [19]. Bacteria were heated at 105°C in a 7.5 *M* solution of sodium hydroxide in methanol–water (6:4, v/v). After acidification (pH 2.0), the liberated fatty acids were extracted with diethyl ether. After evaporation, methyl esters were obtained by reaction with boron trifluoride–methanol solution (20%) and were extracted with chloroform–hexane (1:4, v/v). The organic phase was evaporated under nitrogen and the extracts were stored at −4°C. All the strains were cultivated twice and from each separate culture we made three or four extractions.

Gas chromatography

The lipidic extracts were analysed on a Varian CPG 6000–6500 gas chromatograph equipped with a flame ionization detector. Analysis was performed on a DB1 capillary column (30 m × 0.32 mm I.D., d_f = 0.25 μm) coated with cross-linked methylsilicone. The temperature of the injector and detector was 300°C. The oven temperature was programmed from 80 to 300°C at 4°C/min. Extracts were dissolved in *n*-hexane. The fatty acids, as methyl esters and alcohols, were identified according to their retention times in comparison with those of standard products (a bacterial acid methyl esters mixture, 4-5436; Supelco, Bellefonte, PA, U.S.A.) or by mass spectrometry (VG-Micromass 16 F, VG Analytical, Winsford, U.K.).

SDS-PAGE

Mycobacteria cultures were harvested and suspended in 500 μl of water; the suspension was then treated in an MSE ultrasonic disintegrator for 15 min at a 5-μm amplitude to disrupt the cell walls; during ultrasonic operation the tube was placed in ice. The bacteria were then lyophilized.

SDS-PAGE was performed according to the modified Laemmli method [22] at room temperature in a Pharmacia GE-2/4 LF apparatus. The gel slabs were 140 × 180 mm and 0.7 mm thick. Samples were suspended in buffer to achieve final concentrations of 0.01 *M* Tris–HCl, 0.001 *M* EDTA and 1% SDS. Samples were heated for 5 min at 100°C. The stacking gel buffer was 0.125 *M* Tris–HCl (pH 6.8) containing 0.1% SDS and the separating gel buffer was 0.375 *M* Tris–HCl (pH 8.8) containing 0.1% SDS. The polyacrylamide concentration was 4% for the stacking gel and 12.6% for the separating gel. The electrophoresis buffer was 0.05 *M* Tris solution (pH 8.3) containing 0.384 *M* glycine and 0.1% SDS. The standard proteins (LMW Calibration Proteins 17.0446.01) were obtained from Pharmacia. To visualize protein solutions as an aid to sample migration, bromophenol blue was mixed with the sample. Electrophoresis was performed for 10 min at a constant voltage of 250 V and for 5 h at 150 V. Gels, stained with 0.1% Coomassie brilliant blue solution, were observed on a BIOCOM picture analyser equipped with LECPHOR software for electrophoresis analysis and a COMPAQ computer.

We obtained four or five separate cultures from all the ATCC and type strains, and two or three electrophoreses were performed with each culture.

RESULTS

A representative gas chromatogram for *M. avium* fatty acid methyl esters is shown in Fig. 1A. The corresponding trace for *M. intracellulare* (not shown) was very similar, differing only with minor variations of the relative peak areas. GC does not permit the differentiation of these two related mycobacteria species. Fig. 1B shows the chromatogram obtained with *M. scrofulaceum*. Different extractions made from different separate cultures of the same strain did not show any differences in the chromatograms obtained.

All the strains of the MAIS complex contain the common fatty acids (palmitic, palmitoleic, stearic, oleic, linoleic) and tuberculostearic acid (TSA), a specific compound usually present in mycobacteria. All these constituents are present as methyl esters. Additionally, some other compounds are present in small amounts.

Comparison of Fig. 1A and B shows the presence of an extra peak in the *M. avium* profile. This peak, situated between the C_{19} and the C_{20} saturated acids, represents a specific marker to differentiate *M. scrofulaceum* from *M. avium*/*M. intracellulare*. This additional peak was identified as a secondary fatty alcohol, 2-eicosanol, which is present in all the strains of *M. avium* and *M. intracellulare*; its amount is variable and the peak area may be small.

In order to separate and identify the *M. avium* and *M. intracellulare* species, we applied SDS-PAGE of cell proteins. Fig. 2 shows the electrophoretic patterns corresponding to the three species of the MAIS complex. It represents densitometer tracings of Coomassie brilliant blue-stained gels and a photograph of the electropherogram. The more abundant the protein, the more intense is the col-

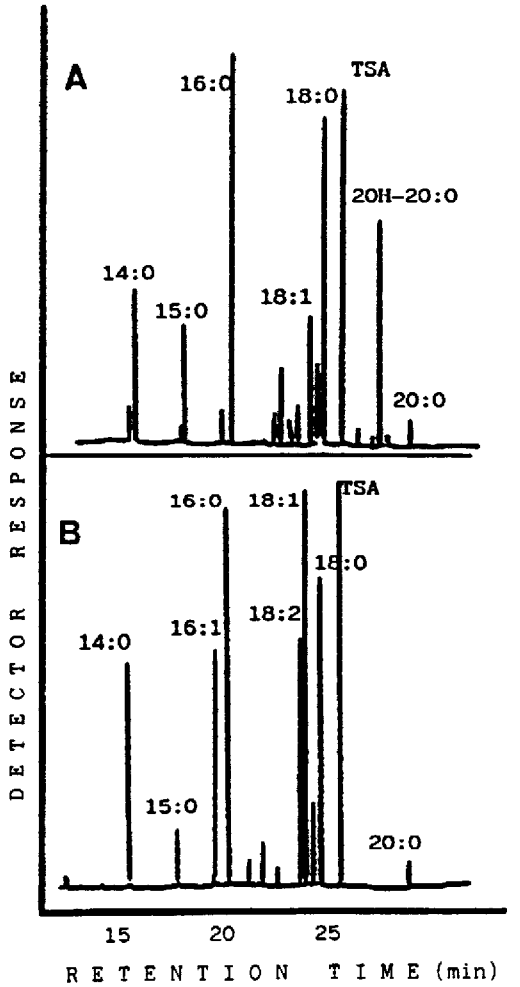

Fig 1 Gas chromatograms of the fatty acids methyl esters of (A) *Mycobacterium avium* and (B) *Mycobacterium scrofulaceum*, ATCC strains. TSA = tuberculostearic acid.

oured band and the darker is the region on the photograph. Table I gives the R_F values, areas and molecular masses of the proteins. The patterns are visually different. The most obvious differences among the strains are seen in the region of the gel corresponding to the proteins of 40 000–50 000 Da and 25 000–30 000 Da relative molecular mass. The different patterns obtained from each strain analysed were similar.

DISCUSSION

Differentiation between the three MAIS complex species is not always evident.

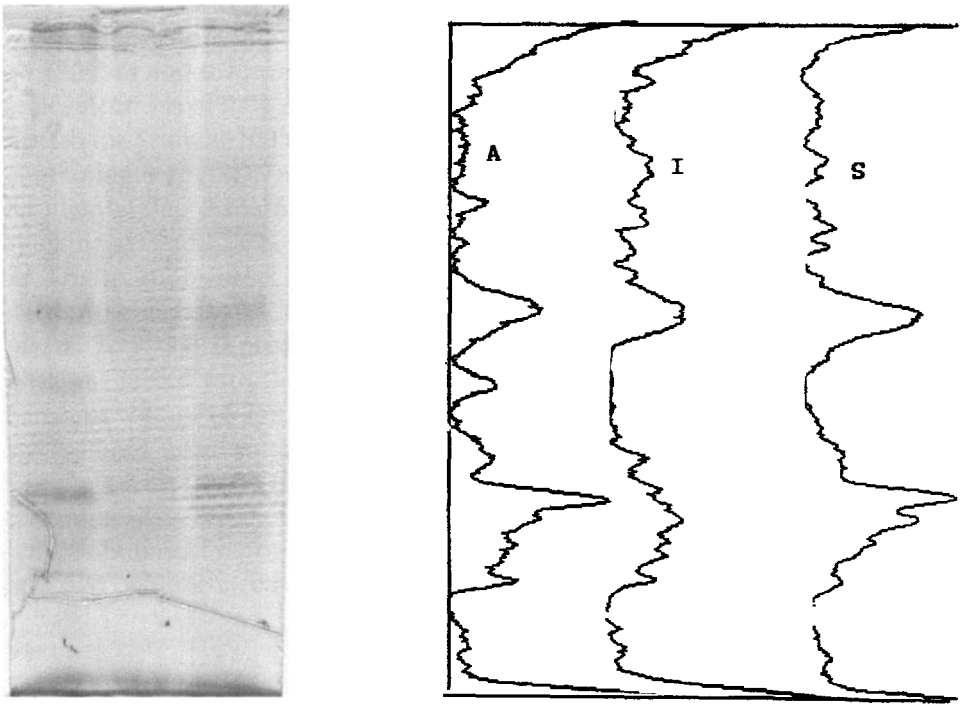

Fig. 2 SDS-PAGE and densitometer tracings of (A) *Mycobacterium avium*, (I) *Mycobacterium intracellulare* and (S) *Mycobacterium scrofulaceum*, ATCC strains.

Some organisms are included in this complex because they are indistinguishable by routine culture methods. Several workers have studied the cellular constituents of mycobacteria using various procedures. One of the most widely used approaches is the analysis of lipid constituents by thin-layer (TLC), gas (GC) or high-performance liquid chromatography (HPLC).

Using GC we were not able to separate *M. avium* from *M. intracellulare* but we identified in this species 2-eicosanol, a secondary fatty alcohol that was not found in the nine *M. scrofulaceum* strains analysed. Hence this alcohol seems to be a discriminating factor. TLC was employed by Daffé *et al* [17] and Jenkins *et al.* [23] to separate the mycolates of these different species; members of the MAIS complex were distinguishable by this procedure. Other workers have tried to discriminate them: neither Tisdall *et al.* [24] using GC nor Butler and Kilburn [16] using HPLC were able to separate *M. avium* from *M. intracellulare*. The presence of 2-eicosanol in *M. avium* has been mentioned in some publications [15,17,25] but it was not used to separate *M. avium*/*M. intracellulare* from *M. scrofulaceum*. Not all the methods used to characterize lipids of mycobacteria by GC yielded the same amounts of fatty alcohols [26]. For this reason we used the techniques described by Larsson *et al.* [25] to verify that *M. scrofulaceum* strains did not

TABLE I

PROTEIN SDS-PAGE OF *MYCOBACTERIUM AVIUM, MYCOBACTERIUM INTRACELLULARE* AND *MYCOBACTERIUM SCROFULACEUM*

R_F, area and molecular mass of different bands. ATCC strains

M avium			*M intracellulare*			*M. scrofulaceum*		
R_F × 100	Area (%)	M_r (Da)	R_F × 100	Area (%)	M_r (Da)	R_F × 100	Area (%)	M_r (Da)
0 5	5.93	119 075	1 7	11 50	116 286	5.6	1 14	107 764
1 2	14.84	117 394	6.4	7.09	106 271	10.5	1 46	97 995
13 4	1.75	92 622	10 5	8 28	98 044	14.0	1 24	91 688
27.6	4.83	70 358	19 3	2 77	82 663	15.4	1 41	89 110
34.0	1.49	62 200	22.0	5.40	78 463	22.8	1 67	77 271
43 5	12.98	51 702	24 2	4 01	75 186	27.9	1.73	69 933
54 0	7 01	42 168	31 1	4 92	65 841	30.4	4 09	66 688
62.6	1.59	35 722	35.2	2.49	60 743	33.1	2.81	63 291
64 5	5 39	34 393	45.0	14.51	50 252	37.5	1.52	58 103
70 4	25 78	30 694	64 5	3 43	34 393	42.6	19.80	52 585
73.1	7.35	29 135	69 2	5 11	31 430	61.0	2.22	36 820
76.8	4.36	27 135	73.8	13.72	28 723	62.5	3.46	35 785
81 9	5 38	24 564	78 7	7.35	26 126	64.2	2.25	34 615
86 8	0 10	22 342	82 2	6 17	24 448	68.9	27.88	31 626
94.1	1.22	19 381	89 2	1 47	21 308	72.3	16.57	29 591
			91.9	1.78	20 226	75.5	5.13	27 818
						81 1	3.90	24 938
						91 9	1.72	20 233

show this supplementary peak in their chromatograms; this method is recommended for extracting fatty alcohols with higher efficiency.

Some workers have considered other cellular constituents for identifying mycobacteria. Genetic relatedness has been suggested for bacterial taxonomy. Electrophoresis of cell proteins has been shown to be of taxonomic value in the classification of mycobacteria [21]. Analysis of the MAIS complex species proteins by SDS-PAGE gave three different patterns. The results in Fig. 2 and Table I demonstrate the differences between the mycobacterial patterns. *M. avium* shows a major protein band which appears at $R_F = 54$ with a molecular mass of 42 168 Da; this protein is missing in *M. intracellulare* and *M. scrofulaceum*. *M. intracellulare* has two bands situated at $R_F = 10.5$ and 22 which are not present in the other two traces. *M. scrofulaceum* shows two major bands with area 27.88 and 16.57% at a molecular mass of *ca.* 30 000 Da. In this region, *M. avium* and *M. intracellulare* have only one prominent band. Hence protein electrophoresis appears to be a means of differentiating mycobacterial species of the MAIS complex; this method reflects genetic differences between species whose culture characteristics may be identical.

GC analysis of cellular fatty acids is a particularly suitable method for the identification of mycobacteria on a routine basis because it is easy to apply and is able to discriminate between closely related species. Protein analysis emphasizes the genetic differences between bacteria and SDS-PAGE is a general method for the separation of strains in epidemiological studies.

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