Detection of 2-Eicosanol by Gas Chromatography-Mass Spectrometry in Sputa from Patients with Pulmonary Mycobacterial Infections

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A total of 96 sputum specimens from patients with suspected or known mycobacterial and nonmycobacterial pulmonary infections were analyzed by gas chromatography-mass spectrometry for the presence of 2-eicosanol. This secondary alcohol was detected in all of the 25 sputum specimens culture positive for Mycobacterium tuberculosis, in 7 of the 9 sputum specimens culture positive for M. avium complex, and in all 3 of the studied sputum specimens associated with M. malmoense. The alcohol was not detected in any of the 45 culture-negative sputum specimens or in 14 sputum specimens culture positive for Staphylococcus aureus, Pseudomonas aeruginosa, Haemophilus influenzae, and Streptococcus pneumoniae. The ratio of tuberculosatic acid to 2-eicosanol was much lower in sputum samples culture positive for mycobacteria than in the corresponding in vitro-grown cultures. The present findings indicate that 2-eicosanol may be useful as a chemical marker for rapid diagnosis of pulmonary infections caused by the M. avium complex, M. malmoense, and M. tuberculosis.

During recent years, there has been an increase in the incidence of infections caused by nontuberculosis mycobacteria. Of these bacteria, Mycobacterium avium complex (MAC) organisms are the most frequently encountered. MAC infections are particularly common in AIDS patients (6). In addition, outbreaks of multidrug-resistant strains of M. tuberculosis have been reported in the United States, and an increased clinical importance of M. malmoense has been found in northern and northwestern Europe (4, 5, 9). Detection of these mycobacteria in clinical specimens usually takes several weeks when conventional methods are used; hence, improved and more rapid diagnostic techniques are needed.

Certain mycobacteria (including MAC) contain bound wax ester mycolates which yield long-chain secondary alcohols, particularly 2-eicosanol, when subjected to hydrolysis (8). We have recently developed gas chromatography-mass spectrometry (GC-MS) methods for the determination of trace levels of these alcohols (2). GC-MS analysis has previously been used to detect small amounts of tuberculosatic acid (TSA) in sputum specimens from patients with mycobacterial infections (11, 14–16).

The purpose of the present study was to investigate whether GC-MS analysis can be used for direct detection of 2-eicosanol in sputum specimens from patients with pulmonary mycobacterial infections. GC-MS results were compared with culturing results, and amounts of TSA found were considered in relation to amounts of 2-eicosanol.

**MATERIALS AND METHODS**

**Chemicals.** Pentfluorobenzyl (PFB) bromide, 1-eicosanol, and 2,3,4,5,6-pentafluorobenzoyl (PFBO) chloride were purchased from Janssen Chemica (Beerse, Belgium), tetrabutylammonium hydrogen sulfate and N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) were from Fluka AG (Buchs, Switzerland), triethylamine was from Sigma (St. Louis, Mo.), pyridine was from E. Merck AG (Darmstadt, Germany), and the solvents acetonitrile, methylene chloride, methanol, and n-hexane were from Lab-Scan (Dublin, Ireland). All chemicals were of analytical grade.

**Mycobacteria.** Two strains each of MAC, M. malmoense, and M. tuberculosis were used. Mycobacterium spp. had previously been isolated from sputum samples, cultivated on liquid Middlebrook 7 H9 medium (M7H9), and identified by both standard biochemical tests and cellular fatty acid analysis (7, 8).

**Sputum specimens.** Forty-two sputum specimens collected from 32 individuals were received at the tuberculosis laboratory of the Department of Clinical Microbiology, Lund Hospital, Lund, Sweden. Seven of the specimens were from four patients with known pulmonary MAC infections. Each of the sputum samples was coded and divided into two equal portions, one of which was used for culturing. The other portion was treated with 2 volumes of dithiothreitol (Sputolysin; Behring Diagnostics, La Jolla, Calif.), homogenized by shaking for 15 min, and centrifuged (15 min at 3,000 × g); the obtained sediment was used for direct GC-MS analysis.

In a second series of experiments, 54 sputum specimens were analyzed: 25 were collected from 15 patients with known pulmonary M. tuberculosis infections, 14 were from 13 patients with nonmycobacterial pulmonary infections, and 15 were from control individuals. All 54 sputum specimens were treated with Sputolysin, and the sediments obtained were divided into two portions, one for culturing and the other for GC-MS.

**Culturing.** Each of the 42 coded specimens was mixed with 2 ml of an aqueous solution containing sodium lauryl sulfate (3%, wt/vol) and sodium hydroxide (1%, wt/vol), shaken for 20 min at room temperature, and centrifuged (15 min at 3,000 × g). The pellet obtained was neutralized with aqueous sulfuric acid (0.45%, vol/vol) and inoculated onto three slants of Löwenstein-Jensen medium (LJ), one of which was supplemented with 0.4 mg of ionizaid per liter (13), and into 10 ml of M7H9. The cultures were then...
incubated at 37°C in a 5% CO₂ atmosphere for 1 week, and thereafter they were incubated in air.

Of the 54 sputum specimens included in the second series of experiments, 32 were cultured for mycobacteria on three plates of LJ as described above. The remaining 22 sputum specimens were cultured for nonmycobacterial organisms on three plates, two horse blood agar plates incubated at 37°C aerobically and anaerobically and one hematin agar plate (containing bacitracin) at 37°C in 5% CO₂.

Sample preparation for GC-MS. The sediments of the Sputolysin-treated sputum samples were sterilized by heating at 100°C for 10 min. The sterilized sediments were then mixed with 1 ml of 30% (wt/vol) methanolic potassium hydroxide and heated at 80°C for 30 min. After being cooled, the alkaline hydrolysate was extracted with hexane. The upper phase was evaporated under a stream of nitrogen and used for PFBBO derivatization of alcohols; the lower phase was acidified with 25% (vol/vol) aqueous sulfuric acid and extracted with hexane for analysis of TSA (as a PFB derivative) as previously described (1).

The cultured mycobacteria (4 mg [dry weight]) were hydrolyzed as described above and analyzed for both TSA and 2-eicosanol by previously described procedures (2).

GC-MS. The GC-MS analyses were performed on a Hewlett-Packard model 5890 gas chromatograph-VG Trio-1S mass spectrometer combination (VG Masslab, Manchester, United Kingdom); both electron impact and negative-ion chemical ionization modes were used. A fused-silica capillary column (25 m by 0.25 mm [inner diameter]) containing crosslinked OV-1 as the stationary phase was interfaced directly to the ion source. Samples (1 μl) were injected in the splitless mode with a Hewlett-Packard model 7673 autosampler. The temperature of the column was programmed from 120 to 270°C at 20°C/min. The temperature of the ion source was 220°C in the electron impact mode and 150°C in the negative-ion chemical ionization mode. The ionization was performed at 70 eV. Helium was used as the carrier gas at a column head pressure of 10 lb/in²; ammonia was used as the reagent gas in the negative-ion chemical ionization mode.

RESULTS

Culturing. Ten of the 42 coded sputum specimens were culture-positive for mycobacteria. By both biochemical tests and GC analyses of the cellular fatty acids and alcohols (7, 9), isolates from nine of these specimens were identified as belonging to MAC, and the isolate from the remaining specimen was identified as M. malmoense. Of the 54 sputum specimens included in the second series of experiments, 39 were culture positive: 25 for M. tuberculosis, 3 for Haemophilus influenzae, 3 for Streptococcus pneumoniae, 7 for Pseudomonas aeruginosa, and 1 for Staphylococcus aureus. These bacteria were identified by standard tests.

GC-MS analysis of sputum. The negative-ion chemical ionization mass spectra of PFBBO-derivatized 2-eicosanol and PFB-derivatized TSA exhibited abundant molecular-specific ions of m/z 492 and 297, respectively; these ions were the focus of the analyses (1, 2). Ten of the 42 coded sputum specimens produced a clearly detectable peak at the retention time of PFBBO-derivatized 2-eicosanol. These 10 sputum specimens were also analyzed for TSA, but the resulting chromatograms were difficult to interpret because of the presence of an unknown compound which eluted close to the TSA derivative (Fig. 1); only 4 of the sputum specimens were clearly positive for TSA.

Of the 54 sputum specimens included in the second series of experiments, all of the 25 specimens culture positive for mycobacteria contained 2-eicosanol, and 23 of these also contained TSA. 2-Eicosanol was not detected in any of the control sputum specimens or the sputum specimens culture positive for nonmycobacterial organisms.

Culturing versus GC-MS. The results of the studies on the coded sputum samples are summarized in Table 1. Both 2-eicosanol analysis and LJ culturing revealed 10 positive sputum specimens, whereas only 7 positives were revealed by M7H9 culturing. Of the 10 2-eicosanol-positive samples, only 4 were TSA positive. Thirty of the 42 studied specimens were negative according to all methods. Two sputum specimens were positive in culture but negative in 2-eicosanol analysis; these spura were from different patients, one known to have had a previous pulmonary MAC infection. Two sputum specimens, positive for 2-eicosanol but negative in culture, were also from different patients, neither of whom was previously known to have had a previous pulmonary mycobacterial infection; one of these sputum specimens was from the same patient who contributed the specimen from which M. malmoense was isolated after 8 weeks of incubation (Table 1). The other 2-eicosanol-positive and culture-negative specimen was from a patient from whom an additional sputum specimen (this specimen was not included in the present study) proved to be culture positive for M. malmoense after 10 weeks of incubation in M7H9 (data not shown).

The ratio of TSA to 2-eicosanol (wt/wt) in the TSA-positive sputum specimens was calculated with known amounts of synthesized TSA and 1-eicosanol as standards. The ratio varied between 0.02 and 0.1 for sputa culture positive for MAC and between 0.2 and 32 for those culture positive for M. tuberculosis. None of the three sputum samples associated with M. malmoense (all of which were 2-eicosanol positive) contained detectable amounts of TSA (Table 2).

GC-MS analysis of cultured mycobacteria. The TSA/2-eicosanol ratios (wt/wt) in two strains of each of the cultured mycobacterial species were 1.2 and 1.6 for MAC, 5.4 × 10² and 7.6 × 10² for M. malmoense, and 2.9 × 10² and 3.5 × 10³ for M. tuberculosis (Table 2). To identify 2-eicosanol in the M. tuberculosis and M. malmoense strains, trimethylsilyl derivatization was also performed; the resulting electron impact mass spectrum exhibited a base ion at m/z 117 and a molecular-specific ion at m/z 355, indicative of 2-eicosanol (2).

DISCUSSION

Infections caused by MAC, M. malmoense, and M. tuberculosis (including drug-resistant strains) represent an increasingly important health problem worldwide. The methods currently used to diagnose these infections are culture based and therefore time-consuming.

The use of GC-MS for direct detection of specific mycobacterial lipid constituents in clinical specimens represents one of several potentially applicable methods for rapid diagnosis of mycobacterial infections. TSA has hitherto been the main analyte used. However, TSA is present not only in Mycobacterium spp. but also in some other members of the family Actinomycetales, and false-positive results of TSA sputum assays for the diagnosis of pulmonary tuberculosis have been reported previously (14–16). In the present study, 4 of 10 sputum samples culture positive for MAC (40%) and 23 of 25 sputum samples culture positive for M. tuberculosis (92%) were TSA positive. These findings correlate with
those of earlier reports that *M. avium* (cultivated in vitro) contains only about 1/10 of the amount of TSA found in *M. tuberculosis* (10).

It has been reported previously that 2-eicosanol is present in a limited number of slowly growing mycobacterial species, including MAC (12). The aim of the present study was to investigate whether this alcohol could be detected directly in sputum specimens by a previously described GC-MS procedure (2). The detection of 2-eicosanol not only in sputum culture positive for MAC but also in sputa associated with *M. malmoense* (Table 1) was unexpected, since, as for the *M. tuberculosis* complex, *M. malmoense* has not been reported previously to contain wax ester mycolates (nor, consequently, secondary alcohols) (12, 17). This finding prompted the second series of experiments in which sputa from patients with pulmonary infections due to *M. tuberculosis* were analyzed. In these experiments, 2-eicosanol was detected in all of the 25 sputum specimens culture positive for this species. Furthermore, the alcohol was identified in cultures of *M. malmoense* and *M. tuberculosis*, although in amounts several thousandfold less than the amounts of TSA. The reason for the dramatic difference between sputum samples and in vitro cultures with regard to TSA/2-eicosanol ratios is not clear, but it may be a result of degradation of phospholipids (containing TSA) in the host. In a study with *M. leprae* isolated from armadillos, it was speculated that observed levels of TSA being low compared with those of mycolates could have been due to a fast catabolism of TSA.

![FIG. 1. GC-MS detection of 2-eicosanol (left panels) and TSA (right panels) in a sputum sample culture positive for MAC (shaded peaks) (upper panels) with corresponding mass spectra (center panels). Chromatograms from the analysis of a control sputum specimen are also shown; arrows indicate the positions of 2-eicosanol and TSA (lower panels).](http://jcm.asm.org/)

### TABLE 1. Detection of MAC and *M. malmoense* in 42 coded sputum specimens by culturing and direct GC-MS analysis

<table>
<thead>
<tr>
<th>No. of specimens</th>
<th>Result by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LJ</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
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<tr>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>-</td>
</tr>
<tr>
<td>Total 42</td>
<td>10</td>
</tr>
</tbody>
</table>

*MAC was cultured for 3 and *M. malmoense* was cultured for 8 weeks. Results are for MAC except as noted. For details about grouping of specimens, see the text.

+ , positive; − , negative; ND, not done.

Only result for *M. malmoense*.

### TABLE 2. Ratios of TSA to 2-eicosanol in cultures and sputum samples

<table>
<thead>
<tr>
<th>Species</th>
<th>Result for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture</td>
</tr>
<tr>
<td>MAC</td>
<td>1.4</td>
</tr>
<tr>
<td><em>M. malmoense</em></td>
<td>6.5 × 10³</td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>3.2 × 10³</td>
</tr>
</tbody>
</table>

* Ratios (wt/wt) were calculated with known amounts of TSA and 1-eicosanol as standards. All results are mean values.
by the enzymes of the animal (3). This may also explain a recently reported low sensitivity of TSA analysis for the detection of M. tuberculosis in sputum (16).

Two of the present sputum specimens which were positive for MAC according to LJ culturing were negative for 2-eicosanol. Clearly, mycobacteria may be unevenly distributed in viscous sputum samples, and we cannot exclude the possibility that dividing the individual specimens for culturing and GC-MS analysis resulted in significantly different mycobacterial proportions in the two specimen parts. By contrast, in the second set of samples which were treated with Sputolysin and homogenized before being divided for GC-MS and culture, 2-eicosanol was detected in all of the 25 sputum specimens culture positive for M. tuberculosis.

Two 2-eicosanol-positive sputum specimens from different patients were culture negative; neither of the patients was previously known to have a mycobacterial infection. However, after the GC-MS results were revealed, additional sputum samples from the same two patients showed growth of M. malmoense. With these findings considered, in the study including the 42 coded sputum specimens the sensitivity of the 2-eicosanol assay was 95% and the specificity was 100%.

The method described here may be useful for the rapid diagnosis of pulmonary diseases due to MAC, M. malmoense, and M. tuberculosis, microorganisms which account for the vast majority of mycobacterial infections in many European countries. Whether 2-eicosanol can be detected in sputum specimens culture positive for other mycobacterial species was not studied. By combining heat killing with alkaline hydrolysis (100°C, 30 min) and omitting Sputolysin treatment, 2-eicosanol analysis results can be provided within 2 h of the specimens being received (data not shown). The present findings indicate that GC-MS analysis of 2-eicosanol may represent an alternative method or a valuable adjunct to other diagnostic methods.

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REFERENCES


