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Journal of Chromatography B, 751 (2001) 143–151

JOURNAL OF CHROMATOGRAPHY B

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Electron-capture gas chromatographic–chemical ionization mass spectrometric study of sera from people vaccinated with bacille Calmette–Guerin for characteristic metabolites

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Received 27 December 1999; received in revised form 5 June 2000; accepted 11 August 2000

Abstract

Serum samples from 26 individuals vaccinated with bacille Calmette–Guerin (BCG) and from 26 controls (10 patients with pulmonary tuberculosis and 16 non BCG-vaccinated healthy individuals) were analyzed by frequency-pulsed electron-capture gas chromatography (FPEC-GC) and chemical ionization gas chromatography–mass spectrometry (CIGC–MS) for the presence of characteristic metabolites. A distinct pattern consisted of tuberculostearic acid (TSA) and a peak, labeled peak 1, was observed in all BCG-vaccinated individuals, whereas only three of 26 controls generated this chromatography profile. TSA was detected in all patients with pulmonary tuberculosis but peak 1 was absent. Sera drawn from 12 individuals 11 to 14 days after BCG vaccination yielded three transitional FPEC-GC profiles. A permanent FPEC-GC profile consisting of TSA and of a full scale peak 1 appeared 28 days to a few months after BCG vaccination. Peak 1 was tentatively identified by CIGC–MS as 9-methyl-hexacosanol. The findings suggest that peak 1 may serve as a marker to detect *Mycobacterium bovis* BCG and to distinguish individuals infected with *M. tuberculosis* from individuals vaccinated with BCG.

Keywords: Bacille Calmette–Guerin; Tuberculostearic acid

1. Introduction

More than 3 billion people worldwide have been vaccinated with bacille Calmette–Guerin (BCG) a non pathogenic strain of *Mycobacterium bovis* [1]. The main reasons for vaccination with BCG are protection against infection with *M. tuberculosis* [2] and for treatment of bladder cancer [3–6]. For vaccination purposes the organism has been introduced into the body by intradermal injection, aerosols, and by mouth [1]. Some of the negative effects of BCG vaccination are as follows: (1) the individual becomes positive for purified protein derivative (PPD) that is used extensively in some countries as an indicator of infection with *M. tuberculosis* [1,2]. (2) The organism has the potential to be pathogenic in immune compromised individuals [2,4,7]. (3) Sera from BCG-vaccinated individuals

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0378-4347/01/$ – see front matter
PII: S0378-4347(00)00465-5

has been reported to contain tuberculostearic acid (TSA) [8]; a fact that could diminish the value of TSA as a potential marker in identifying patients with tuberculosis. Recently, however, during testing of sera from BCG-vaccinated individuals, we found a metabolite labeled peak 1 which appeared to have the potential for use in distinguishing patients infected with *M. tuberculosis* from BCG-vaccinated individuals (unpublished data).

The objectives of this investigation were as follows: (1) to analyze sera of individuals receiving BCG vaccination for TSA and other metabolites, (2) to establish when TSA and peak 1 appear after vaccination, (3) to investigate a possible relationship between peak 1 and the appearance and disappearance of TSA, (4) to gain knowledge about the chemical properties of peak 1, and to establish a frequency-pulsed electron-capture gas chromatography (FPEC-GC) profile that can be used for rapid detection of BCG products in body fluids.

2. Experimental

2.1. Samples

Serum samples used in the study were from residual specimens that had been drawn for routine laboratory testing from healthy controls, from patients with pulmonary tuberculosis and from individuals participating in the BCG vaccination program conducted at the Aghia Sophia Children's Hospital in Athens, Greece. The individuals were vaccinated by intradermal injection of 80 000 to 300 000 attenuated viable bacilli of BCG strain 1077 (Pasteur Merieux, France). The serum samples were given a code number and shipped to US Centers for Disease Control and Prevention (CDC) on dry ice. Clinical information relating to the sample number was available.

2.2. Preparation of trichloroethanol (TCE) esters

The serum sample volume (which ideally specifies 2 ml) ranged from 2 ml to 1 ml from small children. The sample was placed in a 50-ml round-bottomed pyrex centrifuge tube (Corning Inc., Corning, NY, USA), acetified with sulfuric acid to near pH 2, a dash of NaCl (about 20 mg) was added to prevent emulsion, an internal standard consisting of 7 nmol of heptanoic acid was added, 20 ml of nanograde chloroform was added, and the sample was capped with a PTFE-lined screw cap. The sample was immediately extracted using a Burrell wrist action shaker (Burrell, Pittsburgh, PA, USA) at a setting of 5 for 5 min, and concentrated with clean dry air in an 85°C sand bath to near 25μl. Next the sample was derivatized with 10 μl of 1:11 TCE in xylene using 25 μl of heptafluorobutyric anhydride as a catalyst as described [9]. For clean-up of excess TCE following derivatization 100 μl of xylene was added and the sample was evaporated in a sand bath [9] at 85°C using clean dry air filtered through calcium sulfate to almost (but not complete) dryness. Finally, 200 μl of xylene was added as a final solvent, and 2 μl of derivatized sample along with 1 μl of xylene (which was included as a syringe flush) was injected into the FPEC-GC system for analysis.

2.3. Chemical ionization gas chromatography–mass spectrometry (CIGC–MS) analysis

Conformation of trichloroethyltuberculosterate (derivatized TSA) was obtained by a modification of the methane CIGC–MS technique as follows: the retention time obtained from a TCE-derivatized standard of TSA analyzed on a high-resolution 24 m capillary column at 62.05 min was used along with CIGC–MS single ion monitoring (SIM) for the molecular ion at m/z 429 (M+1) and for two chlorine isotopes at m/z 431 and 433 as suggested [8]. The sensitivity for detection of low-fmol concentrations of trichloroethyltuberculosterate using methane CIGC–MS was obtained by adjustment of the mass spectrometer operating conditions for maximum sensitivity, by injection of 4 μl of derivatized sample along with 1 μl of xylene flush, by performing a baseline noise correction using computerized background subtraction of baseline noise taken just prior to elution of TSA, by increasing the mass spectrometer detector voltage to 100 V above the amount specified by CI autotuning of the instrument, and by use of maximum dwell time per ion. The total ion spectrum for identification of peak 1 by CIGC–

MS was obtained by analysis of a sample containing a high concentration of peak 1.

2.4. Operating conditions for CIGC–MS and FPEC-GC

The type OV-1 columns and instrumentation used in the analysis are described [8]. The sample was injected into the FPEC-GC system first and the temperature program started immediately followed by a rapid injection of the CIGC–MS column with the same sample. The instrument was programmed for 4 min isothermally at 90°C then increased for 3°C/min to 285°C and held isothermally at 285°C for 31 min to assure removal of high-boiling compounds that could produce background problems in the following analysis. In the case when a new column was used, the instrument was programmed at 4°C/min instead of 3°C/min until the column was well conditioned. The mass spectrometer was operated under low resolution in the SIM mode with a dwell time of 18 ms for each of the three ions monitored. The dwell time was very important for obtaining the necessary sensitivity. The FPEC-GC analysis was monitored by a COMPAQ DESKPRO XE 560 computer (Compaq Computer, Houston, TX, USA) equipped with a Pentium processor, Dose 6.2 software, and Beckman System Gold software version 8.1 (Beckman, Fullerton, CA, USA), a Hewlett-Packard Laser Jet 4 Plus printer, and a Perkin-Elmer strip chart recorder. Other operating conditions are described [8]. Determination of TSA by FPEC-GC was made by overlaying a TCE-derivatized standard mixture containing C18 (among other acids) and TSA and by aligning C18 with C18 in the sample and reading the matching retention time for TSA.

3. Results

Table 1 presents the chromatography profiles from three different groups of individuals included in the study. All samples from BCG-vaccinated individuals were positive for TSA and yielded a full scale peak 1. Four of 16 of the non BCG-vaccinated healthy controls were positive for TSA and three of them had full scale peak 1, suggesting that these individuals might had been infected with *M. bovis*. Sera from 10 patients with pulmonary tuberculosis were all positive for TSA and negative for the compound labeled peak 1.

Table 2 shows the results obtained by FPEC-GC and CIGC–MS analysis of sera from 12 BCG-vaccinated individuals over time post-vaccination. In individual 1 and 2 serum samples before vaccination were available and used as controls. The grouping patterns (A, B, C, F and H) based on a segment of the FPEC-GC chromatogram from 60 to 84 min also are given in Table 2 and in Figs. 1–3. The grouping was based on detection of or non detection of TSA, peak 1, and the appearance and disappearance of two large tailing peaks referred to as peaks x and y which are possibly associated with an immune response. Group A FPEC-GC pattern was generated by the control group and is shown in Fig. 1A; TSA, P1 and peaks x and y were not detected. Group B pattern appeared 11 to 14 days after vaccination. Group B was similar to group H (Fig. 3A and B) except the amount of TSA was low, but detectable, peak 1 was near half scale, and no peaks x, y, or peak labeled C20 was found. Group C (Fig. 1B) was positive for TSA, peak 1 and a tailing peak y which was just beginning to appear. Group F (Fig. 2A and B and Table 2) was detected from 28 to 66 days. Group F had two large tailing peaks labeled x and y. Because

Table 1
FPEC-GC and CIGC–MS analysis of sera from BCG-vaccinated individuals and control groups

Group	Chromatography profile		
	Peak 1 *n*	TSA *n*	Both *n*
BCG-vaccinated individuals ($n=26$)	26	26	26
Non BCG-vaccinated healthy controls ($n=16$)	3	4	3
Patients with pulmonary tuberculosis ($n=10$)	0	10	0

Table 2
FPEC-GC and CIGC–MS analysis of sera from BCG-vaccinated individuals over time post-vaccination[a]

Individual No., gender/age (y)	Serum sample No.	Time post-BCG (d)	FPEC-GC		CIGC–MS for TSA	FPEC-GC profile
			For P1	For TSA		
Control 1. M/5	TB2283	0	−	−	−	A
Control 2. F/24	TB2334	0	−	−	−	A
1. M/5	TCA5102	14	+	+	+	B
2. F/24	TCA5103	14	+	+	+	B
3. M/26	TCA5104	14	+	+	+	B
4. F/5	TCA5105	14	+	+	+	B
5. F/7	TCA5106	11	+	+	+	B
6. M/5	TCA5107	11	+	+	+	H
7. F/7	TCA5132	11	+	+	+	B
8. F/5	TCA5133	11	+	+	+	B
9. F/3	TCA5134	11	+	+	+	B
10. M/6	TCA5135	11	+	+	+	C
11. M/4	TCA5136	11	+	+	+	C
12. M/8	TCA5137	11	+	+	+	C
1.	TCA5108	42	−	Obscured	+	F
2.	TCA5109	30	+	+	+	C
3.	TCA5110	30	+	+	+	C
4.	TCA5111	28	+	+	+	H
5.	TCA5112	28	+	+	+	H
6.	TCA5113	28	+	+	+	H
7.	TCA5138	28	−	Obscured	+	F
8.	TCA5139	28	+	+	+	H
9.	TCA5140	28	+	+	+	B
10.	TCA5141	28	+	+	+	H
11.	TCA5142	28	+	+	+	H
12.	TCA5143	28	+	+	+	H
1.	TCA5114	66	+	Obscured	+	F
2.	TCA5115	66	+	Obscured	+	F
3.	TCA5116	66	−	Obscured	+	F
1.	TCA5117	150	+	+	+	C
2.	TCA5118	98	+	Obscured	+	F
3.	TCA5119	91	−	Obscured	+	F
2.	TCA5120	118	+	+	+	C
3.	TCA5121	127	+	+	+	H
2.	TCA5122	153	+	+	+	B
3.	TCA5123	156	+	+	+	H
2.	TCA5124	178	+	+	+	H
3.	TCA5125	178	+	+	+	H

[a] FPEC-GC=Frequency-pulsed electron-capture gas chromatography, CIGC–MS=chemical ionization gas chromatography–mass spectrometry, BCG=bacille Calmette–Guerin, TSA=tuberculostearic acid, P1=peak 1, M=Male, F=Female, y=year, and d=day. For an explanation of FPEC-GC profiles A–F see Figs. 1–3.

of these large tailing peaks, TSA could only be detected by CIGC–MS. Peak 1 in the type F sample varied with TSA and could be present either in regular amounts or present in trace amounts depending on the concentration of TSA. This correlation indicated a relationship between TSA and peak 1. FPEC-GC group H (Fig. 3A and B) was the last group to appear after vaccination with BCG. This group was positive for TSA, peak 1 was full scale or above, peaks x and y were not present, and a peak labeled by the computer as C20 was present. The peak labeled C20 was tentatively determined by CIGC–MS to be a C30 alcohol. After the appearance of group H the FPEC-GC profile remained un-

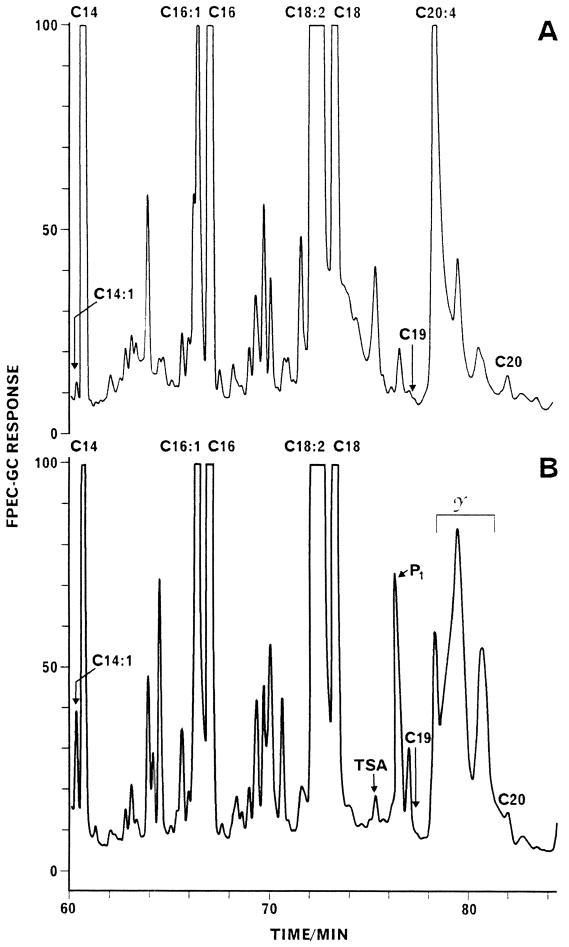

Fig. 1. (A) Chromatogram made from a frequency-pulsed electron-capture gas chromatography (FPEC-GC) analysis of an acetic chloroform extraction and derivatization with trichloroethanol (TCE). A partial chromatogram (60 to 85 min) of a control serum TB2334 which was taken prior to vaccination with bacille Calmette–Guerin (BCG) is shown. This type of chromatogram is referred to as a type A FPEC-GC profile. (B) A similar FPEC-GC chromatogram made from a serum several days after vaccination with BCG. TSA and peak 1 are present and a clump of peaks eluting after C19 can be seen beginning to emerge. This type of FPEC-GC profile is referred to as a type C profile. The analysis was made on a large bore capillary column, 25 m×0.5 mm I.D. coated with 5 μm thick OV-1. The instrument was programmed at 90°C for 4 min, then for 3°C/min to 285°C and held isothermally at 285°C for 31 min. The electron-capture detector was 15-mCi ^{63}Ni. The carrier gas was helium at a flow-rate of 5 ml/min.

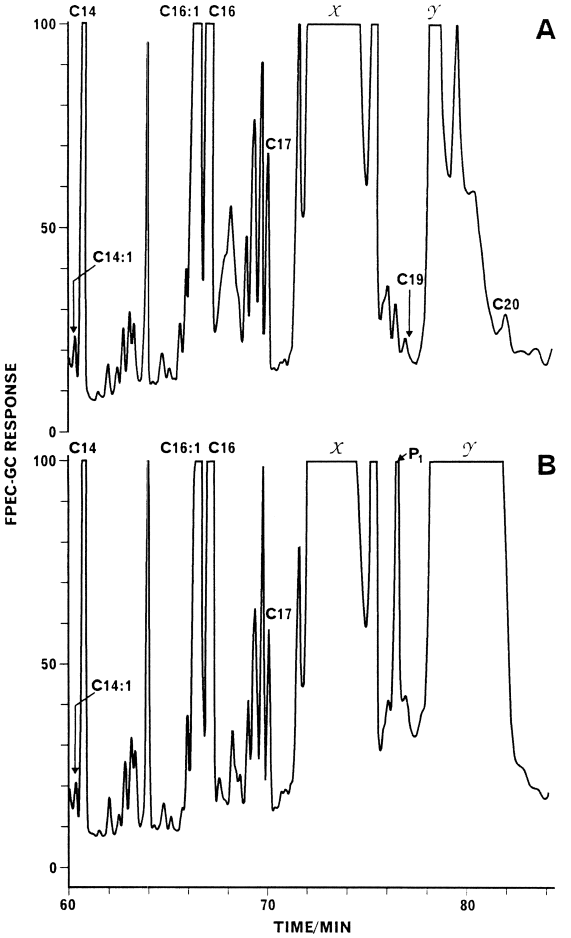

Fig. 2. FPEC-GC chromatograms of two patients 66 days after vaccination with BCG. The FPEC-GC chromatograms illustrate type F profiles. Observe that peak 1 is very low or missing in chromatogram A, and CIGC–MS showed that TSA was also present in low concentration. In chromatogram B the individual (TCA5114) has a full scale peak 1 and CIGC–MS analysis shows that the serum was positive for TSA.

changed, and it was detected in a serum sample 43 years after vaccination with BCG. The detection of TSA and peak 1 produced evidence that BCG may remain alive in the vaccinated individuals for a long period of time after vaccination which is supported by a review of the world literature [10] indicating that BCG provides protection for 10 or more years.

The CIGC–MS spectra showing the molecular ion at m/z 429 (M+1) and two chlorine isotopes of trichloroethyltuberculosterate at m/z 431 and 433 are shown Fig. 4. Eleven to 14 days following BCG vaccination eight of the 12 vaccinated individuals had a type B FPEC-GC grouping pattern, one (TCA5107) had a type H pattern, and three

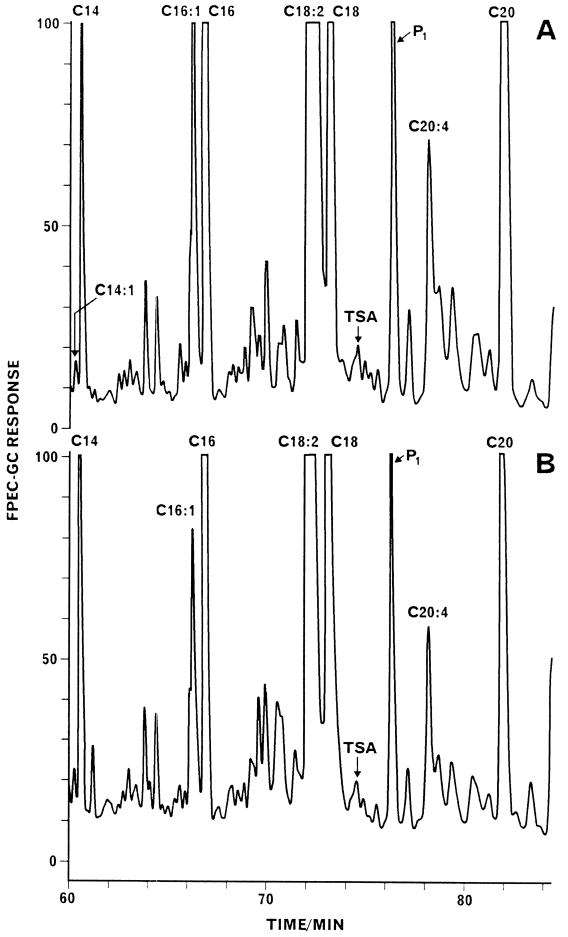

Fig. 3. FPEC-GC chromatograms from the same individuals shown in Fig. 2, 156 and 178 days post BCG vaccination. FPEC-GC profiles have changed from type F (Fig. 2) to type H over time post-vaccination.

(TCA5135, TCA5136 and TCA5137) had a type C pattern. From the type B FPEC-GC pattern the individuals usually proceeded to a type C to F and finally to a type H FPEC-GC pattern (Table 2 and Fig. 3A and B). The FPEC-GC pattern that has been developed over time in the serum of individuals vaccinated with BCG clearly illustrates the association of TSA and P1 with BCG. Neither TSA nor peak 1 were detected before vaccination (Fig. 1A, Table 2). TSA and peak 1 were detected in small amounts 14 days post-vaccination (Table 2), and peak 1 was full scale 156 and 178 days post-vaccination, pattern H (Table 2 and Fig. 3A and B).

Fig. 5 shows the complete CIGC–MS spectra obtained from the analysis of peak 1. Peak 1 was tentatively identified from the CIGC–MS spectra as a HFBA derivative of a C27 alcohol (1-heptafluorobutryl-9-methyl-hexacosonol) with the possibility of a branched chain methyl group. The branch is suggested because of a fragmentation in the chain at the ninth carbon atom. The molecular ion is missing as often is found in HFBA-derivatized long-chain alcohols as determined by the analysis of a variety of HFBA-derivatized alcohol standards (unpublished data). Derivatization with HFBA is detected in the fragments at m/z 169, 197 and 213. The molecular ion, if detected by CIGC–MS, would be located at m/z 591 (M−1). The fragment at m/z 379 was formed by a splitting of the chain and loss of 213 (the heptafluorobutryl portion) to leave the remainder of the molecule at m/z 379. The next fragment at m/z 241 was formed by a split at the branched carbon to lose the fragment H-·C-(CH_3)-CH_2-(CH_2)$_6$-CH=CH_2 and to leave the remainder of the molecule CH_3-$(CH_2)_{16}^{+2H}$ at m/z 241.

4. Discussion

It was shown herein that sera from BCG-vaccinated individuals yield a distinct chromatographic pattern consisted of TSA and of a compound labeled peak 1. These findings may have important implications in distinguishing BCG-vaccinated individuals from individuals infected with *M. tuberculosis*. They also provide some information on the symbiotic relationship between BCG organisms and the host.

Indeed, FPEC-GC and CIGC–MS analysis of sera revealed different chromatographic profiles in BCG-vaccinated individuals and in patients with active pulmonary tuberculosis; a characteristic TSA–peak 1 pattern was generated in the former group, whereas only TSA in small quantities was detected in the latter. On the contrary, in individuals with latent infection (PPD positive healthy individuals), tubercle bacilli have minimal or no metabolic activity and TSA is not detected, as has been reported previously [8,9,11,12]. This important difference in the chromatographic profile may be useful in distinguishing PPD positive individuals due to BCG vaccination

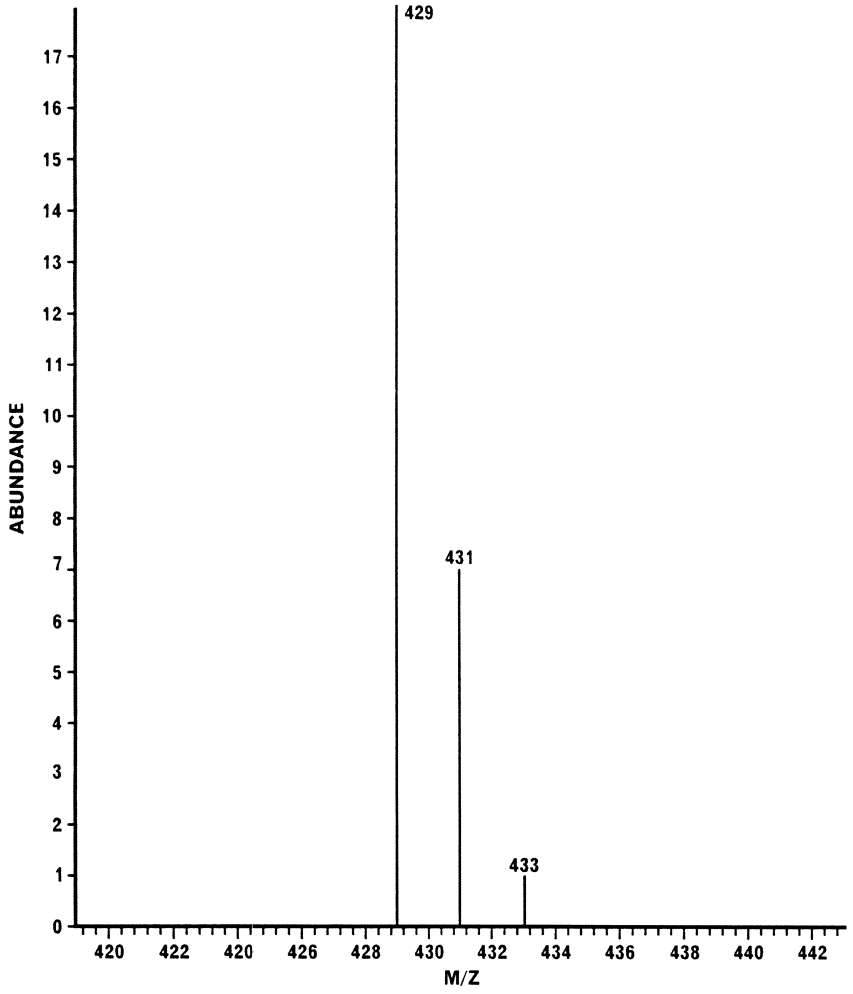

Fig. 4. Methane chemical ionization single ion monitoring spectra obtained from the acetic chloroform TCE-derivatized extract of serum. The serum sample had a type F FPEC-GC profile. The molecular ion at m/z 429 (M+1) along with two chlorine isotopes at m/z 431 and 433 are shown.

from PPD positive healthy individuals due to exposure to *M. tuberculosis*.

Although the TSA–peak 1 pattern was characteristic for BCG-vaccinated individuals, this pattern was observed in three non BCG-vaccinated healthy controls. It is not known, however, whether these individuals had acquired BCG organisms by some type of close contact with a person vaccinated with BCG. A prior infection with *M. bovis* could also explain this chromatographic profile.

TSA and peak 1 were detected in the sera of BCG-vaccinated individuals soon after vaccination. In some cases TSA and peak 1 were reduced significantly after the appearance of peaks x and y possibly due to reduction of BCG organisms by the host's immune response. Both compounds, however, reappeared with time. The concurrent reduction and reappearance of TSA and peak 1 suggest a relationship between these two compounds and possibly reflect the multiplication state of the organisms. A permanent chromatography profile appeared 28 days to a few months after vaccination. The consistent detection of both TSA and peak 1 in all BCG-vaccinated individuals suggests that the bacilli are

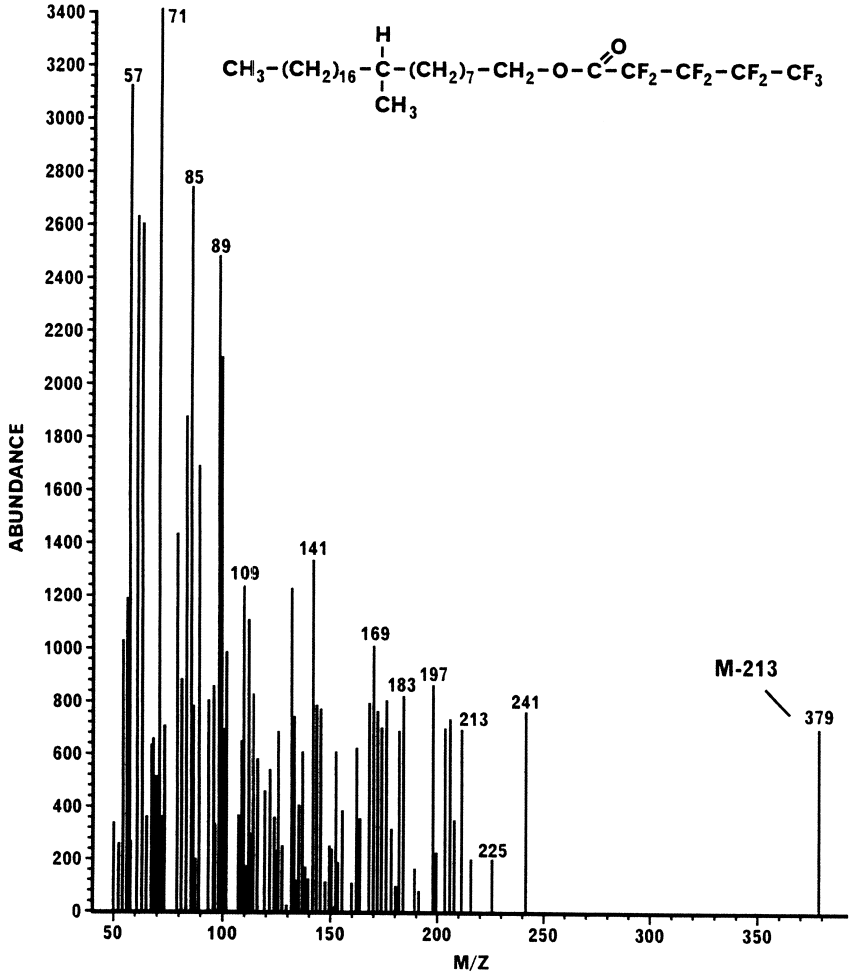

Fig. 5. Total ion CIGC–MS spectra of peak 1 obtained from an individual positive for peak 1. The chromatogram was obtained from a TCE-derivatized extract using heptafluorobutyric anhydride (HFBA) as a catalyst. The spectra indicates that peak 1 is a HFBA derivative of a branched chain C27 alcohol (1-heptafluorobutryl-9-methyl-hexacosanol).

not eradicated by the host but they multiply and survive for prolonged period of time.

The use of FPEC-GC is essential for identification of peak 1 and other peaks in the chromatographic profile. Use of CIGC–MS adds confidence to the analysis of TSA, and it can be used to identify TSA in the presence of a tailing peak that may obscure detection by FPEC-GC. It may be possible to add CIGC–MS SIM monitoring for peak 1 to the CIGC–MS identification system. Use of CIGC–MS, however, would increase the cost.

Peak 1, which was found in sera from people vaccinated with BCG, was tentatively identified in the derivatized form using CIGC–MS as 1-heptafluorobutryl-9-methyl-hexacosanol. Whether heptacosanol will be found also in the sera from clinical cases of pathogenic *M. bovis* remains to be investigated. If so, the detection of heptacosanol might become a valuable means to distinguish *M. tuberculosis* from *M. bovis* infections. Moreover, heptacosanol could provide a long awaited marker to tract pathogenic *M. bovis* and BCG in vivo. Animal models might be useful for determining differences and similarities between the two organisms in vivo, and they could be tested by FPEC-GC and CIGC–MS before exposure to the organism, after exposure,

and after therapy. However, well documented human cases will eventually have to be tested for possible differences in human and animal models.

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