Mycolic Acid Patterns of Representatives of *Mycobacterium bovis* BCG

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(Received 7 June 1984)

The mycolic acids of 15 cultures of representative *Mycobacterium bovis* BCG substrains were examined by alkaline hydrolysis followed by esterification and thin-layer chromatography of the resulting methyl esters. Representatives of the Moreau and Swedish substrains had mycolic acid patterns similar to *M. bovis*, composed of α -mycolates, methoxymycolates and ketomycolates, but examples of the Pasteur, Glaxo, Prague, Danish and Chinese substrains did not contain methoxymycolates. This apparent subdivision of *M. bovis* BCG substrains is in accordance with their varying antigenic patterns recorded in other studies.

INTRODUCTION

Mycolic acids are characteristic high molecular weight 3-hydroxy-2-alkyl branched fatty acids whose distribution is of value in mycobacterial systematics (Minnikin & Goodfellow, 1980; Minnikin, 1982; Dobson *et al.*, 1984). In a previous study (Minnikin *et al.*, 1983) it was found that only one of four vaccine strains of *Mycobacterium bovis* BCG had the expected pattern of mycolates characteristic of *M. bovis*, methoxymycolates being absent in the remaining three strains. In the present study representative cultures were examined to test whether the simplified mycolic acid pattern was characteristic of a wider selection of *M. bovis* BCG variants.

METHODS

Strains. The organisms examined are listed in Table 1. In Copenhagen the 11 MNC strains were cultivated on Sauton's medium at 38 °C for 4-6 weeks. Cultures were sterilized in flowing steam for 1 h, harvested by filtration and dried at about 80 °C overnight. The remaining four GA and GB strains were grown in Göteborg on Sauton's medium at 37 °C for 42 d. After harvesting by filtration, organisms were killed by treatment with a final concentration of 0.35% formaldehyde solution and freeze-dried. The cultivation of these four strains was duplicated using modified Sauton's medium in which 6% (v/v) glycerol was replaced by 1.5% (w/v) glucose and 2% (w/v) casein hydrolysate.

Mycolic acid analysis. Dried biomass was degraded by alkaline hydrolysis followed by phase-transfer catalysed conversion to methyl esters (Dobson *et al.*, 1984) and long-chain compounds were examined by two-dimensional TLC (Minnikin *et al.*, 1980, 1983). The total mycolic acid methyl esters were isolated by preparative TLC, converted to *t*-butyldimethylsilyl (TBDMS) ethers and analysed by TLC using petroleum ether (b.p. 60-80 °C)/toluene (70:30, v/v) in a single direction (Dobson *et al.*, 1984).

RESULTS AND DISCUSSION

The TLC patterns of long-chain compounds extracted from the test organisms cultivated on Sauton's medium are shown in Fig. 1, which also includes a duplicate culture of GB 453 using Sauton's medium modified with glucose as carbon source. The TLC patterns for GB 295,

Table 1. *Mycobacterium bovis* BCG substrains used

Laboratory no.*	Strains and comment	Source†
MNC 5	BCG vaccine, BCG department, SSI‡. Received 1954	K. Tolderlund
MNC 448	J. Stanford 101, Glaxo. Received 1973	G. Kronvall
MNC 654	L. Šula BCG A. Received 1964	L. Šula
MNC 655	L. Šula BCG K. Received 1964	L. Šula
MNC 656	L. Šula BCG R. Received 1964	L. Šula
MNC 657	BCG Dal Brazil, Moreau. Received 1964	A. Jespersen
MNC 1241	BCG vaccine batch no. 72, BCG department, SSI. Received 1977	I. Baess
MNC 1329	CIPT 14 004 0001 BCG Institut Pasteur, Paris. Received 1980	H. David
MNC 1395	BCG vaccine, freeze-dried lot 445, BCG department, SSI. Received 1982	K. Bunch-Christensen
MNC 1403	BCG Chinese strain batch 007. Received 1982	K. Bunch-Christensen
MNC 1408	BCG Swedish strain, seed lot 2, freeze-dried 1971. Received 1983	A. Ladefoged
GA 001	BCG Swedish strain, seed lot 1, freeze-dried 1965. Received 1926	A. Calmette
GB 295	International working reference, batch no. 3. Received 1981	K. Bunch-Christensen
GB 453	BCG Swedish strain, seed lot 2, freeze-dried 1971. Received 1983	K. Bunch-Christensen
GB 454	BCG Danish strain, no. 1331. Received 1983	K. Bunch-Christensen

* MNC, M. Magnusson; GA and GB, A. Lind and M. Ridell.

† Dr I. Baess, Copenhagen, Denmark; Dr A. Calmette (deceased), Paris, France; Dr K. Bunch-Christensen, Copenhagen, Denmark; Dr H. David, Paris, France; Dr A. Jespersen (deceased), Copenhagen, Denmark; Dr G. Kronvall, Addis Ababa, Ethiopia; Dr A. Ladefoged, Copenhagen, Denmark; Dr L. Šula, Prague, Czechoslovakia; Dr K. Tolderlund (deceased), Copenhagen, Denmark.

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GB 454 and GA 001 grown on modified Sauton's medium were practically indistinguishable from those shown for these organisms in Fig. 1. The identity of the mycolic acid esters was confirmed by TLC of their TBDMS ether derivatives (Dobson *et al.*, 1984).

The majority of the *M. bovis* BCG samples (Table 1, Fig. 1) produced the simplified mycolic acid pattern composed of only ketomycolates and so-called α -mycolates which lack oxygen functions in addition to the 3-hydroxy acid unit. The exceptions (Fig. 1) were the Brazilian Moreau substrain (MNC 657) and the Swedish substrains cultivated both in Copenhagen (MNC 1408) and Göteborg (GB 453 and GA 001), which also contained a methoxymycolate. These results provide confirmation of the patterns recorded previously (Minnikin *et al.*, 1983) for Moreau, Glaxo, Prague and Pasteur substrains. In an independent study the result for the Pasteur substrain was confirmed (Daffé *et al.*, 1983). A Russian BCG substrain was considered to have a mycolic acid composition similar to that for the Moreau and Swedish BCG substrains and that expected for *M. bovis* (Asselineau & Portelance, 1974; Daffé *et al.*, 1983). The absence of methoxymycolates in trehalose mycolates ('cord-factors') from *M. bovis* BCG Pasteur substrain (Adam *et al.*, 1967; Strain *et al.*, 1977) is also explained.

The present results show some correlation with other comparative studies on BCG substrains. Early immunodiffusion analyses showed that the Swedish, Moreau and Russian strains contained an antigen which was not demonstrable in the Danish and French strains (Lind, 1960). Cultivation of the Swedish substrain on the bromocresol purple medium, used for the differentiation of *M. tuberculosis* and *M. bovis*, gave no colour change, as found in *M. bovis*; the Danish substrain resulted in a change from violet to yellow (Meissner, 1968). The Roumanian BCG substrain and the Moreau, Japanese and Russian substrains have been found to share an antigen not detected in the Danish, French and Prague substrains (Stavri *et al.*, 1981). A characteristic antigenic protein, termed MPB70, was detected in the Japanese Tokyo and Brazilian Moreau BCG substrains (Miura *et al.*, 1983). Skin reactions on guinea pigs showed that this protein was present in much smaller amounts in the French BCG substrain, and that it was apparently not present in the Danish and Chinese Beijing substrains (Miura *et al.*, 1983). Two-dimensional electrophoresis of culture filtrates showed that MPB70 was present in *M. bovis* Ravenel and absent in *M. tuberculosis* H37Rv and Aoyama B (Miura *et al.*, 1983). By use of more sensitive radioimmunoassay inhibition tests, high concentrations of MPB70 were found in the Tokyo, Moreau, Swedish and Russian BCG substrains and in *M. bovis* Ravenel, while a hundred

Fig. 1. Two-dimensional TLC of long-chain compounds from BCG strains (Table 1); all organisms were grown on Sauton's medium except for GB 453*, which was grown on modified Sauton's with glucose and casein hydrolysate. A triple development with petroleum ether (b.p. 60–80 °C)/acetone (95:5, v/v) in the first direction was followed by a single development with toluene/acetone (97:3, v/v) in the second direction. Abbreviations: A, α -mycolate; B, methoxymycolate; C, ketomycolate; F, non-hydroxylated fatty acid methyl esters.

times lower concentrations, or less, were detected in the Danish, French, Glaxo and Chicago Tice BCG substrains and in *M. tuberculosis* H37Rv (Harboe & Nagai, 1984).

It is apparent that two groups of BCG substrains are becoming discernible with one group containing the French Pasteur, British Glaxo, Danish Copenhagen, Czechoslovakian Prague, Chicago Tice and Chinese BCG substrains and the other containing the Brazilian Moreau, Japanese Tokyo, Russian, Swedish and Roumanian BCG substrains. Methoxymycolates and protein MPB70 are practically absent in representatives of the former group but present in some of the latter and in *M. bovis*; further studies will be necessary to prepare a complete set of data for these taxonomic characters. The latter group appears to be closer in character to the original parent *M. bovis* species, though the virulence of the Swedish and Moreau strains, for example, is still low (Jespersen, 1971). It is not possible to judge, on the present evidence, if representatives

of the latter group have reverted in the direction of *M. bovis* or if those in the former group have moved further from Calmette's original BCG strain during the years of continuous subculturing (Rosenthal, 1980). The analysis of mycolate patterns appears to have potential for the reliable assignment of BCG daughter strains to one or other of the two groups. Other chemotaxonomic characters such as fatty acid profiles and complex lipid patterns (Minnikin & Goodfellow, 1980; Minnikin, 1982) should be analysed to test whether they support the apparent subdivision of BCG substrains.

J. H. P. was supported by a grant (G8216538) from the Medical Research Council to D. E. M. and M. Goodfellow, and M. R. and A. L. were supported by a grant from the Swedish National Association against Heart and Chest Diseases.

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