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Subtyping of *Mycobacterium avium* complex (MAC) isolates by thin-layer chromatography – distribution of subtypes from patients with AIDS compared with clinically non-significant isolates

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SUMMARY

Thin-layer chromatography (TLC) was compared with seroagglutination for subtyping of *Mycobacterium avium* complex (MAC) bacteria. Seventy-five significant MAC isolates from patients with AIDS were typed by both methods and 36 isolates, judged to be clinically non-significant, were examined by TLC only. Overall, 75% of isolates tested were typable by seroagglutination and 91% by TLC; the results correlated between the two except for minor discrepancies.

Serovars 1, 8 and 21 and mixed serovars 1–21 and 1–8–21 were common among isolates from AIDS patients and together represented 83% of isolates compared with only 36% in the non-significant group (odds ratio 8·4; 95% confidence interval 3·4–23·3). This difference remained significant after exclusion of serovar 41 (*M. scrofulaceum*), which was the commonest isolate (28%) in the non-significant group but was not isolated from patients with AIDS.

TLC is useful to supplement seroagglutination for subtyping of MAC. Further study is required to determine whether apparent differences between isolates from patients with AIDS and from other sources reflect differences in virulence or in environmental prevalence of MAC subtypes.

INTRODUCTION

The *Mycobacterium avium* complex (MAC) has been recognized in recent years as among the commonest opportunistic pathogens in patients with AIDS [1]. The number of MAC isolates referred to the New South Wales Mycobacterium Reference Laboratory at the Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital, has increased over the past 5 years from 75 in 1987 to 269 in 1992. More than 90% are isolated from patients with AIDS. Conventional biochemical tests are used to differentiate MAC from other slowly growing non-tuberculous mycobacteria but special methods are required to distinguish members of the complex. Various subtyping techniques have been described, including seroagglutination [2, 3], thin-layer chromatography [3, 4], plasmid analysis [5–7], gas chromatography–mass spectrometry [3] and restriction fragment length polymorphisms [8].

Serotyping has been used previously to examine the distribution of AIDS-related MAC isolates in Australia, but is limited by the high proportion of non-typable isolates [9]. Serovar-specific antigens are polar C-mycosidic glycopeptidolipids (GPLs) which can be separated and differentiated from each other by thin-layer chromatography [4]. The aim of this study was to evaluate thin-layer chromatography (TLC) as a method of subtyping of MAC and to compare the distribution of subtypes among isolates from patients with AIDS with that among other human isolates in New South Wales.

MATERIALS AND METHODS

MAC isolates

Seventy-five MAC isolates from patients with AIDS and 36 isolates judged to be not clinically significant (presumed colonizing or contaminant strains) were examined. Only one isolate from each subject was included; those from patients with AIDS were from blood (14 isolates), bone marrow (23), other normally sterile sites (5), faeces (26), and respiratory specimens (7) and the presumed non-significant isolates were from respiratory specimens (32) and urine (4). All were identified as belonging to MAC by conventional biochemical tests. The 75 isolates from patients with AIDS had been serotyped previously by David Dawson, Queensland State Health Laboratories, who also supplied a panel of known MAC serovars, including 1–22, 26 and 41–43.

Growth and storage of mycobacteria for TLC

Organisms grown on Middlebrook's 7H11 agar were suspended in distilled water and the turbidity adjusted using a Macfarland No. 1 standard tube. Two drops were inoculated on to each of three culture plates containing 7H11 agar with OAD and spread evenly using a bent glass stick. The plates were sealed in plastic bags and incubated at 35 °C for 3 weeks. This usually provided sufficient growth of MAC for TLC. After incubation, the mycobacterial colonies were scraped from the surface of the agar, transferred to preweighed (Kimax) culture tubes with Teflon-lined screw caps (13 × 100 mm) and freeze-dried in the presence of nitrogen to prevent degradation of lipid components by lipases (which can occur even at −20 °C). They were then stored under nitrogen until extraction.

Lipid extraction and deacetylation

Chloroform–methanol (Analar, BDH) – 2:1, vol/vol; 32 ml/g of bacteria – was added to dried bacteria in Kimax tubes. The tubes were placed in a sonic bath (Biosonik, Bronwill) for 5 sec; lipids were extracted by incubation at 50 °C for 18 h. Tubes were then centrifuged at 1500 g for 30 min. The clear supernatant was aspirated and stored at 4 °C in tubes with tightly fitting Teflon-lined lids.

Lipid extracts were subsequently incubated with an equal volume of NaOH (0·2 M) in methanol (500 μl) at 37 °C for 20 min; the mixture was neutralized with 12·5 μl of glacial acetic acid and dried under a stream of nitrogen at 37 °C. Chloroform–methanol (2:1, 2·5 ml) then water (500 μl) were added to the dry

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residue, vortex-mixed and centrifuged (1500 g for 30 min) to allow partitioning of non-lipid contaminants into the aqueous phase, which was then discarded. The chloroform phase was dried in a stream of nitrogen as before and the residue dissolved in 500 μl of chloroform–methanol (2:1). This essential step removes acetyl groups of constituent sugars and destroys unwanted neutral lipids and phosphoglycerides without affecting C-mycosidic glycopeptidolipids (GPLs) [4].

Thin-layer chromatography

The method used was essentially that of Brennan and colleagues [4]. Briefly: thin-layer plates (20 × 10 cm × 0·25 mm) were coated with silica gel 60, dried in a hot air oven, cleaned with acetone, and activated at 110 °C for 30 min before use. Samples (20 μl) of the deacylated lipid extracts were applied to the plate. Three developing solvents were used with different proportions of chloroform, methanol and water by volume as follows: solvent I, 60:27:4; solvent II 65:25:4; solvent III 60:16:2. Butylated hydroxytoluene (Sigma Chemical Co.), 0·005% (w/v) was added to the solvents, to prevent autoxidation of fatty acids. Solvents were poured into a filter-paper-lined tank (Twin Trough Chamber, CMAG) to a depth of 1·5 cm and covered for at least 1 h to allow equilibration of the contents with the vapour. The plates were inserted and developed until the solvent front had moved a distance of 15–17 cm from the origin. They were then dried in air, sprayed with 0·1% orcinol in 40% H_2SO_4 and heated for 5–7 min at 110 °C to produce the yellow-gold colour characteristic of 6-deoxyhexoses, the sugars present in C-mycosidic (polar) GPLs. The distance of each GPL from the origin was measured and expressed as a fraction of the distance moved by the solvent front (relative fraction).

RESULTS

Each serovar developed a characteristic profile of one, two or three polar GPLs when separated by TLC. Fast moving serovars, e.g. 1, 2, 4, 6, 12, 14, 15 and 42, were separated by solvent I. More slowly moving serovars, e.g. 9, 10, 11, 21 and 26, stayed near the origin in solvent I but were separated by solvent II, in which the faster moving serovars travelled with the solvent front and could not be differentiated. Resolution of some lipids was only achieved by comparison of profiles in solvents II and III. The serovar pairs 18/19, 21/26 and 22/43 were difficult to distinguish using solvent I but could be differentiated with solvent II. More than one GPL was demonstrated for serovars 5, 16, 20, and 41 in solvent I and for serovars 5, 9, 10, 11, 12, 17, 21 and 22 in solvent II. Fig. 1 shows the relative fraction (Rf) values of lipid profiles of 26 standard control MAC serovars in solvents I (Fig. 1a) and II (Fig. 1b).

Results of seroagglutination were available for 75 MAC isolates from patients with AIDS; 56 were typable and the results are shown in Table 1. Fifty of these 56 isolates were identified by TLC as the same (single) serovar; the remaining 6 were identified as combined serovar 1–8–21 by seroagglutination and as 1–21 by TLC. Of the 19 (25%) strains which were autoagglutinable or non-typable by seroagglutination, 16 were identified by TLC as serovar 1 (6 isolates) or combined serovar 1–21 (10 isolates). Only 3 isolates (4%) were non-typable by TLC. The commonest MAC serovars isolated from patients with AIDS were 1, 8, 21 and

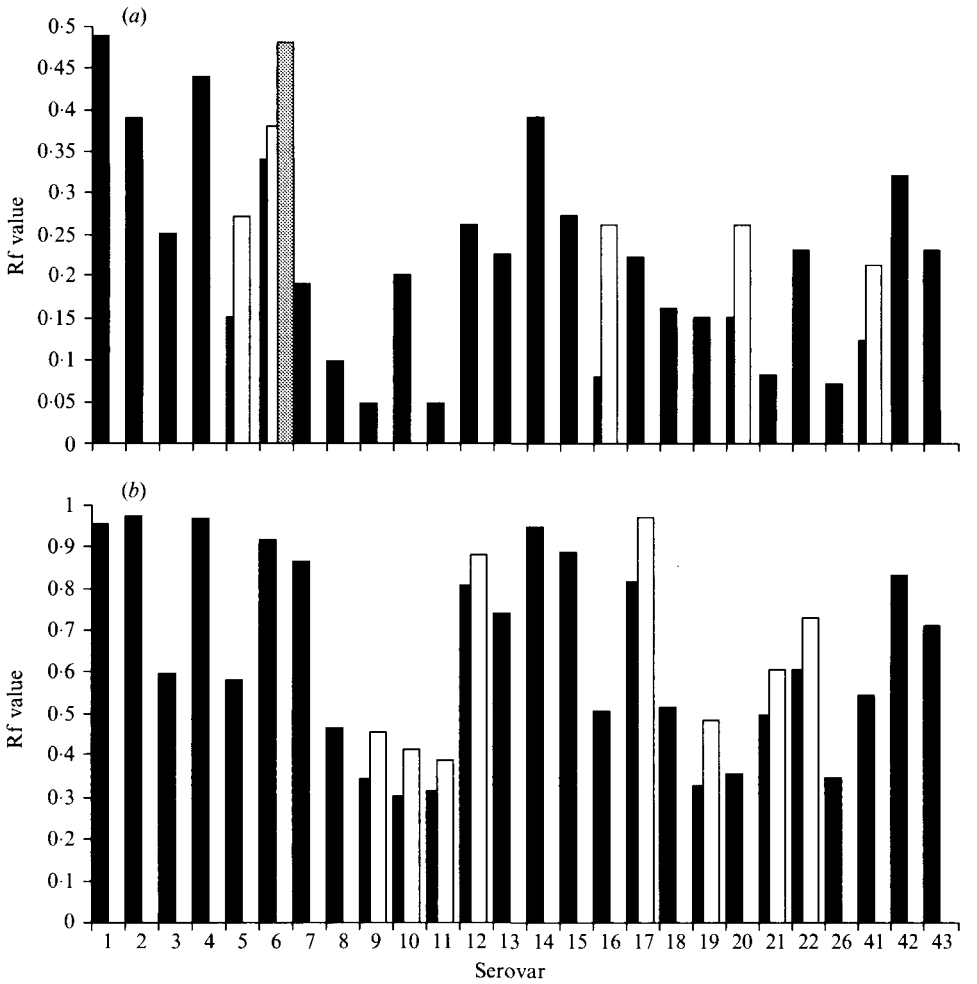

Fig. 1. Relative fraction (Rf) diagrams for lipid thin-layer chromatography profiles of control *M. avium* complex serovars: Fig. 1a in solvent I: Fig. 1b in solvent II. Note similarities between serovar pairs 18/19, 21/26 and 22/43 in 1a which are easily distinguishable in 1b. Multiple bars for some serovars indicate that more than one glycopeptidolipid (GPL) was demonstrable. Mixed serovars (such as 1–21 and 1–8–21) were identified by the presence of GPLs corresponding with all or both individual serovars.

combinations of them (1–21 or 1–8–21). Thirty-six clinically non-significant MAC isolates were subtyped by TLC only. As shown in Table 1, the commonest serovar identified was 41, followed by serovar 1; none in this group was identified as serovar 21. Six isolates (17%) were non-typable.

Overall, 83% (62/75) of isolates from AIDS patients expressed 1 or more of the 3 antigens, 1, 8 and 21, compared with 36% (13/36) of the non-significant ('environmental') isolates. This difference was highly significant (odds ratio 8·4. 95% confidence interval 3·1–23·3) and remained so when serovar 41 (*M. scrofulaceum*) isolates were excluded (13/26; odds ratio 4·8. 95% confidence interval, 1·6–14·2).

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Table 1. *Subtyping of* M. avium *complex isolates by seroagglutination and thin-layer chromatography (TLC)*

	Isolates from patients with AIDS		Other isolates
Method	Seroagglutination	TLC	TLC
Serovar	*n* (%)	*n* (%)	*n* (%)
1	14 (19)	20 (27)	6 (17)
2	5	5	4
4	—	—	3
8	18 (24)	18 (24)	4 (11)
9	3	3	—
14	1	1	—
16	1	1	—
21	8 (11)	8 (11)	—
1–8–21/1–21	6	16 (21)*	3 (8)
41	—	—	10 (28)
Non-typable	19 (25)†	3	6
Combined serovars			
1, 8, 21, 1–21 & 1·8–21	38 (51)	62 (83)‡	13 (36)‡
Total	75	75	36

* All serovar 1–21 by TLC, including 10 non-typable strains and 6 typed as 1–8–21 by seroagglutination.
† Autoagglutinable or non-agglutinating.
‡ $P < 0{\cdot}00001$ (chi square test): odds ratio 8·4 (95% confidence interval 3·1–23·3).

DISCUSSION

The *Mycobacterium avium* complex (MAC) includes 28 serovars. Based on species-specific DNA probes, serovars 1–6, 8–11 and 21 are *M. avium*, 7, 12–20 and 25 are *M. intracellulare* and the others remain unclassified [10]. *M. scrofulaceum*, previously grouped with MAC, is now recognized as a distinct species [11] and usually recognizable by pigment production in the dark. Non-pigmented strains are indistinguishable from MAC by conventional methods but can be differentiated by serotyping, as serovars 41–43 [2].

The prevalence of human infection with MAC varies in different geographical areas but, since the start of the AIDS epidemic, it has become the commonest non-tuberculous mycobacterium associated with human disease [10]. Previously, it usually caused chronic respiratory infection in patients with underlying respiratory disease. However, in 40–50% of patients with AIDS, disseminated MAC infection occurs within 2 years of diagnosis [10, 12] and, without treatment, survival is significantly reduced [13]. The incidence is inversely proportional to the CD4 count and 93% of cases occur in patients with CD4 counts below $100 \times 10^9/l$ [12]. Clinical features include fever, weight loss, anaemia and diarrhoea; the gastrointestinal tract is apparently the usual portal of entry and respiratory infection is uncommon. Almost all isolates from AIDS patients with disseminated MAC infections are *M. avium*, where clinically significant MAC isolates from non-AIDS patients with respiratory infection include a significant proportion which are *M. intracellulare*, as determined by the use of species-specific probes [14].

MAC can be isolated commonly from natural and artificial water sources and soil but the prevalence and serovar distribution vary in different geographical

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areas [15]. In some studies, apparent differences in the distribution of subtypes between isolates from different clinical sources and the environment suggest that some MAC strains are more virulent [5, 8, 16, 17] but others have not demonstrated such differences [7]. In this study isolates, identified as MAC by biochemical tests, from patients with AIDS were compared with clinically non-significant isolates assumed to be representative of environmental strains.

Serovars 1, 8 and 21 and mixed serovars 1–8–21 and 1–21 were all found more frequently among isolates from AIDS patients in New South Wales than among the non-significant isolates. The relationship of these mixed serovars to each other and to serovars 1, 8 and 21 is unknown. There is antigenic cross-reactivity between serovars 8 and 21, although they are easily distinguishable by TLC. Overall, isolates expressing one or more of these three antigens were significantly more common among AIDS-associated than 'environmental' isolates. The possibility that they share common virulence determinants warrants further investigation. Only two isolates were *M. intracellulare* serovars, both from patients with AIDS. Serovar 41 (*M. scrofulaceum*) was the commonest of the non-AIDS-associated isolates but was not found among those from patients with AIDS although other serovars in this group were reported among isolates from AIDS patients in Sweden [18].

A previous study in which seroagglutination was used to type MAC isolates from AIDS patients in Australia (which included the 75 isolates used in the present study), showed significant geographical variation [9]. In Victoria, 41% of isolates were serovar 4 and serovars 1 (12%) and 8 (11%) were less common; in New South Wales only 11% were serovar 4 and serovars 1 and 8 were the commonest isolates (20 and 26% respectively). A greater proportion of AIDS patients in Victoria (95/238, 40%) were reported to have had MAC infection than in NSW (85/768, 11%), suggesting that serovar 4 accounted for most of the excess. In the USA, serovar 4 was predominant in New York (51%) and San Francisco (42%) and serovar 8 in Los Angeles (36%) [17].

Differences in the distribution of subtypes from AIDS patients compared with those from non-AIDS patients or from healthy subjects have been described. In one study, serovar 4 was found significantly more often among AIDS-associated isolates but there were differences in geographical origins of AIDS and non-AIDS-associated isolates [16]. In another, 73% of isolates from AIDS patients and 39% from non-AIDS patients were a single RFLP type which was not found among isolates from healthy subjects [8]. In the USA isolates from AIDS patients are reported to be more likely to harbour plasmids, suggesting an association with increased virulence [5]. However, this was not confirmed by workers in the United Kingdom, suggesting that geographical differences may be more important [7]. The latter observations have been supported by smaller studies of serotype distribution in Europe showing geographical differences [18, 19].

Further study is required to determine whether observed differences between MAC isolates from patients with AIDS and those from other sources represent differences in virulence, environmental distribution or both. Although MAC bacteria are apparently ubiquitous in the environment, differences in their overall prevalence and in the distribution of serovars could explain differences in the incidence of disseminated MAC infection in different geographical locations. Some environmental reservoirs of MAC, such as potable water [20], may be amenable to

control if exposure could be shown to be correlated with an increased risk of MAC infection in those at risk. A suitable method of subtyping is required for further studies of epidemiology and virulence of MAC. Although TLC would allow differentiation of a greater proportion of isolates than seroagglutination, genetic methods of subtyping are more likely to demonstrate relationships between serotypes which are apparently associated with increased virulence.

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