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Cytometric Detection of Mycobacterial Surface Antigens: Exposure of Mannosyl Epitopes and of the Arabinan Segment of Arabinomannans

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The physical arrangement of cell envelope components leads to the exposure of selected structural motifs which in turn may influence host-parasite interactions. To gain insight into the exposed epitopes, the present study describes a flow cytometric method designed to probe defined molecules on dispersed mycobacteria. The hydrophobic fluorophore *N*-hexadecanoyl aminofluorescein inserted in the mycobacterial cell envelope permitted focusing of fluorescence-activated cell sorter analysis on cells that were further labeled with defined monoclonal antibodies and fluorochrome-coupled streptavidin. The use of antibodies directed against the lipooligosaccharide of *Mycobacterium tuberculosis* demonstrated the specific detection of the antigen on the cell surface of a Canetti-like strain of *M. tuberculosis*, and not on those of mycobacterial strains that were devoid of the glycolipid. Thus, the method was applied to investigate the relative amounts of surface-exposed mannosylated compounds and D-arabinan-containing substances of different strains of the tubercle bacillus and a strain of the rapidly growing nonpathogenic species *Mycobacterium smegmatis*. Both *M. tuberculosis* and *M. smegmatis* are endowed with mannosyl and arabinan epitopes on their surfaces, although there are many differences in terms of exposed mannosyl epitopes between the various strains of the tubercle bacillus examined. These differences are correlated with the amounts of terminal mannosyl residues that cap the surface-exposed arabinomannans (A. Ortalo-Magné, A. B. Andersen, and M. Daffé, Microbiology 142:927–935, 1996) but not with the degrees of virulence of the strains. This novel approach could provide new insights into the distribution of defined surface-exposed antigens and thereby into the architecture of the cell envelopes.

The mycobacterial world includes some of humanity's oldest and most feared microbial pathogens, such as the etiological agents of tuberculosis and leprosy and some major opportunistic pathogens that complicate other important human diseases. The increase in the incidence of tuberculosis worldwide, added to the appearance of multi-drug-resistant tubercle bacilli, has renewed interest in studying these acid-fast organisms (3, 5). Pathogenic mycobacteria, with the possible exception of the leprosy bacillus, are facultative intracellular parasites that are capable of surviving and multiplying in phagocytes. This success is most likely inherent in the unusual properties of the mycobacterial cell envelopes. That mycobacteria are appreciably more resistant to deleterious agents, such as acids, alkalis, and germicides (36), also supports this concept. In addition, several cell envelope components have been shown to impair host immunological responses (1, 4), further substantiating the implication of mycobacterial cell envelopes and their components in the pathogenesis of mycobacterial infections. Schematically, these envelopes are composed of three entities: a plasma membrane; a cell wall skeleton consisting of two covalently attached macromolecules, peptidoglycan and mycoloyl arabinogalactan (AG); and an outermost layer (25). As far as the chemical nature of the envelopes is concerned, no interspecies variation has been observed in the molecular composition or the structural features of either the cell wall skeleton or the plasma membrane (10, 13, 25–28). In contrast, it has been noted that pathogenic mycobacterial species differ from the nonpathogenic ones by the presence of a thick electron-transparent outermost layer, also called a capsule, which surrounds each bacterium (19) and which may be part of the defense mechanism permitting these pathogens to resist killing by phagocytic cells (15).

The molecular composition of the latter structure in various mycobacteria has recently been reinvestigated and was defined as mainly consisting of proteins and polysaccharides (24, 30). These studies did not address, however, the question of the arrangement of the different constituents at the cell surface. In addition, it is the physical arrangement of the cell surface which determines what part of the various components comes into contact with the host cells or is recognized by the immune system (14). In the context of tuberculosis, it has been shown that the surface-exposed arabinomannans (AMs) (29, 30) and their lipidated forms (20) isolated from the different strains of the tubercle bacillus are mostly capped with mannosyl residues whatever the virulence of the strains (7, 21, 24, 29, 32, 35); the molecules may, however, differently arrange their structural motifs in such a way that the mannosyl segments of the polysaccharides of virulent strains will be exposed at the surface, whereas those of the avirulent strains will be buried in the inner envelope compartment. Such an arrangement could then explain the different phagocytosis processes that occur between virulent and avirulent strains of *Mycobacterium tuberculosis* (34). Consequently, we adapted the flow cytometric technique for analysis of selected surface-exposed antigens of mycobacteria.

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MATERIALS AND METHODS

Strains and growth conditions. *Mycobacterium bovis* BCG (Pasteur 1173P2), *M. tuberculosis* (Erdman [ATCC 35801], H37Rv [ATCC 27294], H37Ra [ATCC 25177], R1Rv [ATCC 35818], R1Ra [ATCC 35819], and CIPT 140010060 [a Canetti-like strain]), and *Mycobacterium smegmatis* (ATCC 607) were grown on Sauton's medium (33) (100 ml per flask) as surface pellicles at 37°C.

Purification of antigens. The phenol glycolipid (PGL-tb-1 [11]) and lipooligosaccharide (LOS-II [12]) of *M. tuberculosis* were purified from the crude lipid extract of the Canetti-like strain by column chromatography as described previously (11, 12). Similarly, the species-specific diacyl trehalose (DAT [22]), the ubiquitous monomycoloyl trehalose (MMT), and dimycoloyl trehalose (DMT) were isolated from *M. smegmatis*. PGL-K-1 was isolated from *Mycobacterium kansasii* (17). AM (29, 31), lipoarabinomannan (LAM [35]), and phosphatidylinositol mannosides (PIMs [16]) were purified from *M. tuberculosis* H37Rv, as described previously (16, 29, 35); AG of *M. bovis* BCG was isolated from purified cell walls (11).

Immunological procedures. (i) MAbs. Monoclonal antibodies (MAbs) raised against T lymphocytes (L3T4; Tebu; Le Perray-en-Yvelines, France), LAM (L9 [18]), PGL-tb-1 (III604 [8]), PIM (L4 [16]), and LOS-II of *M. tuberculosis* (SNCho [9]) were used; the MAb directed against LOS-II was obtained by a procedure similar to that applied to PGL-tb-1 (8). In addition, a MAb (2D3E10) reacting with nondefined mycobacterial cell wall carbohydrate antigens was generously supplied by M. Blanchard (Bio Atlantic, Nantes, France). By using various anti-mouse immunoglobulins (Igs [polyvalent, anti-IgG1, anti-IgG2a, anti-IgG2b, anti-IgG3, and anti-IgM]; Sigma; L'Isle D'Abeau Chesnes, France), the MAb was identified as belonging to the IgM type; a titration curve revealed that the best dilution of the MAb was 1:100 (by volume in phosphate-buffered saline [PBS]).

(ii) ELISA. Lipids were suspended by sonication at concentrations of 10 μg/ml in ethanol; 10 to 50 μl was applied to the wells of a polystyrene microtiter plate (Nunc-Immuno Plate I; Nunc, Roskilde, Denmark) and allowed to evaporate at 37°C overnight. The wells were saturated with 200 μl of a 3% (wt/vol) bovine serum albumin (BSA) solution in PBS at 37°C for 2 h and washed three times with PBS. The MAbs were diluted (1:10 or 1:100, by volume) in PBS containing 0.3% (wt/vol) BSA and added to the plates, followed by a 2-h incubation at 37°C. After five further washings, 100 μl of alkaline phosphatase conjugated to whole anti-mouse Igs diluted 1:100 (by volume) in 0.3% (wt/vol) BSA in PBS was added, followed by a 2-h incubation at 37°C. After washing of the plates five times with PBS, 100 μl of *p*-nitrophenyl-phosphate substrate (Sigma) was added, followed by a 30-min incubation at 37°C. A_{405} was measured with a Multiskan apparatus (Flow Laboratories, Inc., McLean, Va.).

(iii) Inhibition ELISA. The inhibition of the reaction between 0.6 μg (2×10^{-4} μmol) of solid-phase lipid extract of DMT and MAb 2D3E10 diluted 1:10 (by volume) in PBS by different mycobacterial antigens and sugars was performed as follows. The inhibitory compounds were suspended at various concentrations in PBS. Next, 45 μl of these suspensions and 5 μl of MAb 2D3E10 were deposited in wells that had previously been coated with DMT and blocked by 200 μl of PBS containing 5% (wt/vol) BSA. The mixture was left for 2 h at 37°C before rinsing. The colored reaction was obtained with anti-mouse Igs and *p*-nitrophenyl-phosphate substrate (both from Sigma) as described above, and then the A_{405} was recorded.

A control for no inhibition was performed with 5 μl of MAb diluted 1:10 (by volume) in PBS and 45 μl of the buffer; a blank control was obtained by the development of the complete ELISA reaction in wells not coated with DMT. Each value was measured in triplicate.

Labeling of mycobacteria for flow cytometric analysis. All experiments were performed in a MSC III safety cabinet. Cells were declumped by a gentle shaking with glass beads, as previously described (31), and single cells were recovered in the turbid supernatant, washed with PBS containing 1% (wt/vol) BSA, and harvested by centrifugation (13,000 × *g*, 5 min). The pellets were resuspended in 100 μl of PBS, and 0.5 μl of a 15 mM dimethylformamide solution of 5-*N*-hexadecanoyl aminofluorescein (HEDAF; λ emission, 520 nm; Molecular Probes, Eugene, Oreg.) was added to the suspensions. After a 30-min incubation at room temperature, 1 ml of PBS containing 1% (wt/vol) BSA was added to the reaction mixtures, and the bacterial suspensions were centrifuged as described above. The supernatants were discarded, and the pellets were washed three times with the same buffer to obtain nonfluorescent supernatants. The washed pellets were then resuspended in 1 ml of PBS.

HEDAF-labeled cells (20 μl) were applied to the wells of a polystyrene microtiter plate (Nunc-Immuno Plate I) and centrifuged (13,000 × *g*, 5 min). The supernatants were discarded, and the pellets were suspended in 100 μl of MAbs (L4 or L9) for 1 h at 4°C. The nonspecific binding of the MAbs to the mycobacterial cells was determined with an irrelevant IgG3 (hamster anti-mouse CD4α) MAb. The reaction mixtures were centrifuged and then washed twice with 1 ml of PBS, and the pellets were resuspended in 50 μl of biotinylated goat anti-mouse polyclonal antibodies (Amersham, Les Ulis, France) diluted 1:100 (by volume) in PBS for 1 h at 4°C. After centrifugation and washing, treated cells were incubated with 50 μl of a Tri Color-coupled streptavidin (λ emission, 667 nm; Tebu) for 30 min at room temperature. The resulting bacteria were rinsed with PBS and suspended in 300 μl of the same buffer, and HEDAF and Tri Color double-labeled cells were analyzed per experiment.

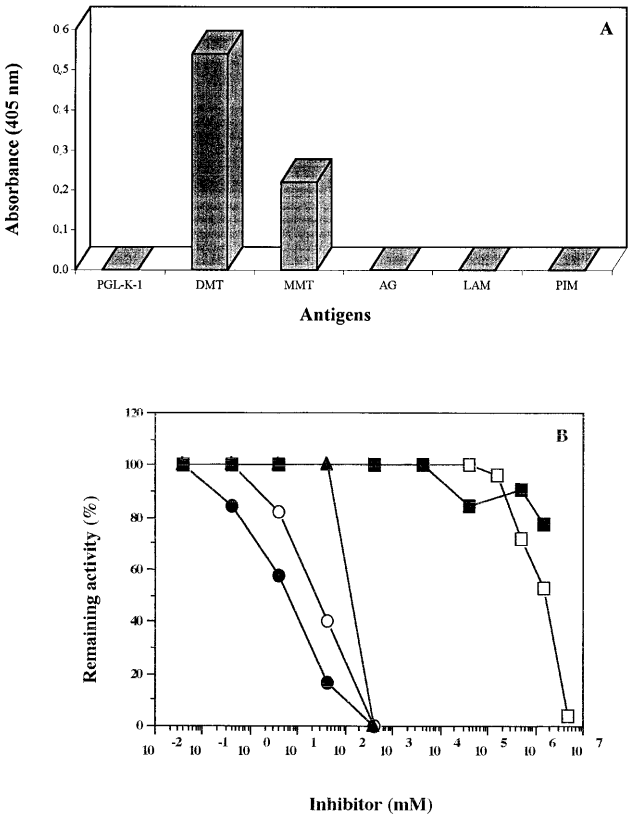

FIG. 1. Reactivity in ELISA of the anti-ATA MAb (diluted 1:100, by volume, in PBS) with defined antigens (0.5 μg) (A) and inhibitory activity of the reaction between the MAb (diluted 1:10, by volume, in PBS) and 0.6 μg of coated DMT by the following inhibitors (B): DMT (●), MMT, (○), DAT (▲), methyl α-D-Glc (□), and methyl α-D-Man (■).

Flow cytometric analysis. All experiments were performed with a FACScan (Beckton Dickinson Immunocytometry Systems, San Jose, Calif.) fluorescence-activated cell sorter. The mycobacterial cell morphological region was first defined in terms of size (forward angle light scatter) and of granularity (light side scatter). Cells positive for HEDAF were gated on an FL 1-labeled window and then analyzed for the presence of Tri Color-coupled streptavidin with a 630-nm FL 3 filter. The fluorescence intensities of 5,000 cells (mean fluorescence intensities) were recorded on a logarithmic scale and analyzed with Lysis II software and an HP9000 series 340 computer station (Hewlett-Packard).

RESULTS AND DISCUSSION

Characterization of the MAbs. The MAb 2D3E10 reacted with mycobacterial sonicates; furthermore, the MAb also recognized the lipids that composed the organic phase of a chloroform-methanol-water (3:4:3, by volume) partition of the whole lipid extracts. These data indicated that MAb 2D3E10 recognizes glycolipids. Thus, the reactivity of the MAb with purified glycolipid antigens was probed by ELISA (Fig. 1A). Among the glycoconjugates tested, the ubiquitous trehalose derivatives reacted strongly with the MAb. Furthermore, a competitive ELISA with coated DMT and suspensions of DAT, MMT, and various methyl glycosides as inhibitors showed that the latter two glycolipids specifically inhibited the binding of DMT to the MAb (Fig. 1B) but were less efficient than DMT itself in inhibiting the binding. Among the standard methyl glycosides tested for inhibiting the reaction, α-methyl glucoside was the most effective competitor compared with β-methyl glucoside (data not shown), proving the crucial role in the anomeric configuration; in addition, α-methyl manno-

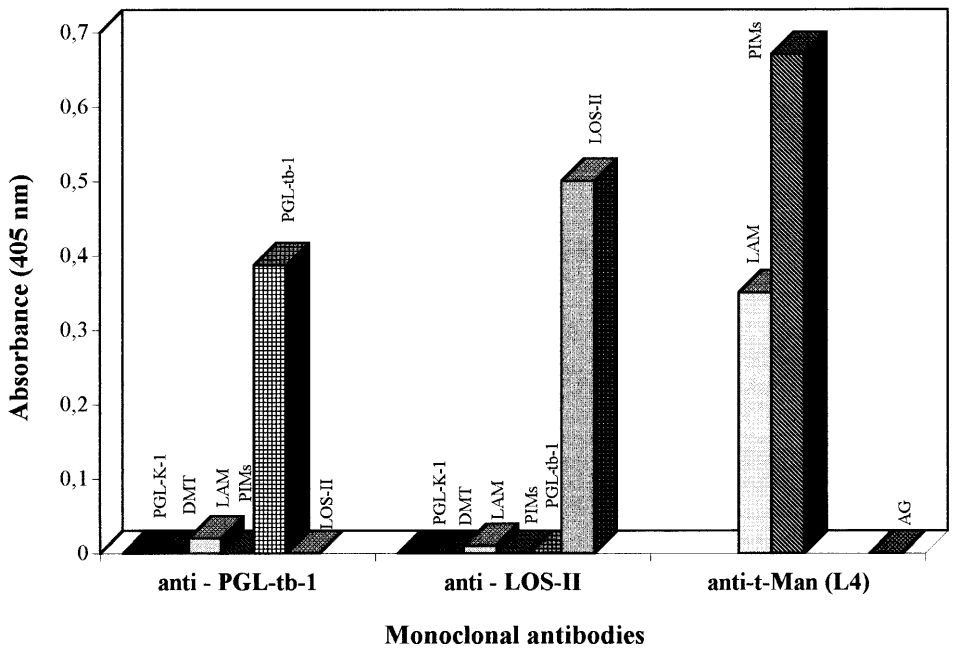

FIG. 2. Reactivity in ELISA of three MAbs with defined antigens (0.5 μg): the major phenolic glycolipid of *M. kansasii* (PGL-K-1), the DMT of *M. smegmatis*, the LAM of *M. tuberculosis* H37Rv, the PIMs of *M. tuberculosis* H37Rv, the major PGL of *M. tuberculosis* Canetti (PGL-tb-1), the antigenic LOS of *M. tuberculosis* Canetti (LOS-II), and the AG of *M. bovis* BCG. The anti-PGL-tb-1 and anti-LOS MAbs were diluted 1:100 (by volume) in PBS, and the anti-t-Man MAb (L4) was diluted 1:10 (by volume) in PBS.

side (Fig. 1B), β-methyl galactoside, and inositol (data not shown) did not significantly inhibit the binding, pointing to the importance of the configuration of C-2, C-4, and C-6, respectively. As expected from the structure of the reacting antigens, α, α′-trehalose was five times more efficient than α-methyl glucoside in inhibiting the binding of the MAb to DMT. Therefore, the binding of 2D3E10 to the acyltrehalose antigens (ATA [i.e., DMT, MMT, and DAT]) involves the C-1, C-2, C-4, and C-6 of the α-glucosyl residue.

Although the specificities of some of the MAbs used in the present study have been previously defined (8, 16), it was important to reinvestigate their reactivity toward some of the dominant mycobacterial antigens that have recently been shown to be located on the cell surface, such as AMs (24, 30). The MAbs that were raised against the PGL-tb-1 and LOS-II of *M. tuberculosis* (8, 9) specifically reacted with the corresponding antigens (Fig. 2), whereas L4, which recognizes (2-OH) unsubstituted mannosyl epitopes (16), reacted with the ubiquitous PIMs, as expected, but also reacted with the LAM of *M. tuberculosis* (Fig. 2). Because the latter MAb did not react with AG, it follows that the MAb recognizes terminal mannosyl residues (t-Man), including those characterized on mannose-capped LAM of slowly growing mycobacterial species (7, 21, 32, 35). We previously showed that the L9 MAb was directed against the D-arabinan segments of both LAM and AG (30). The specificities of the various MAbs used in the present study are summarized in Table 1.

TABLE 1. MAbs used in this study and their specificities

MAb	Specificity	Source or reference
L3T4	Anti-CD4α	—[a]
2D3E10	Anti-ATA	This study
SNCho	Anti-LOS-II	This study (8)
III604	Anti-PGL-tb-1	This study (9)
L9	Anti-arabinan	30
L4	Anti-t-Man	This study (16)

[a] —, commercially available MAb.

Labeling of mycobacterial cells for cytometric analysis. Although flow cytometric analysis has been widely applied to various organisms, it was important to adapt the method to the analysis of mycobacteria, which are known to form large clumps. Thus, the specific detection of individual mycobacterial cells was needed. For this purpose, single bacterial populations were obtained by a gentle mechanical treatment of clumped cells with glass beads, a method known to preserve the integrity of bacteria (31). The declumped cells were then labeled with HEDAF, a hydrophobic fluorescent probe (λ emission, 520 nm) that is expected to insert into the mycobacterial cell envelope, which is known to be highly rich in lipids. Next, labeled cells were coated with selected MAbs. These MAbs were revealed with biotinylated anti-mouse antibodies, which in turn were labeled with another fluorescent probe, fluorochrome-coupled streptavidin (Tri Color; λ emission, 667 nm). The resulting bacteria were then analyzed by flow cytometry.

Bacteria were first gated in a well-defined morphological region (R2 [Fig. 3A]) in which cells stained by HEDAF were selected with FL 1 (window R1 [Fig. 3B]). The HEDAF-labeled bacteria were then analyzed for the presence of the MAb by Tri Color with FL 3 (Fig. 3B), and the FL 3 fluorescence intensities of the double labeled cells were measured in the log mode.

Detection of the selectively distributed LOS-II, PGL-tb-1, and ATA on the cell surfaces of mycobacteria. The fluorescence intensities of the different labeled mycobacterial strains were determined for the various MAbs used (Table 2). In contrast to the unrelated MAb raised against T cells (anti-

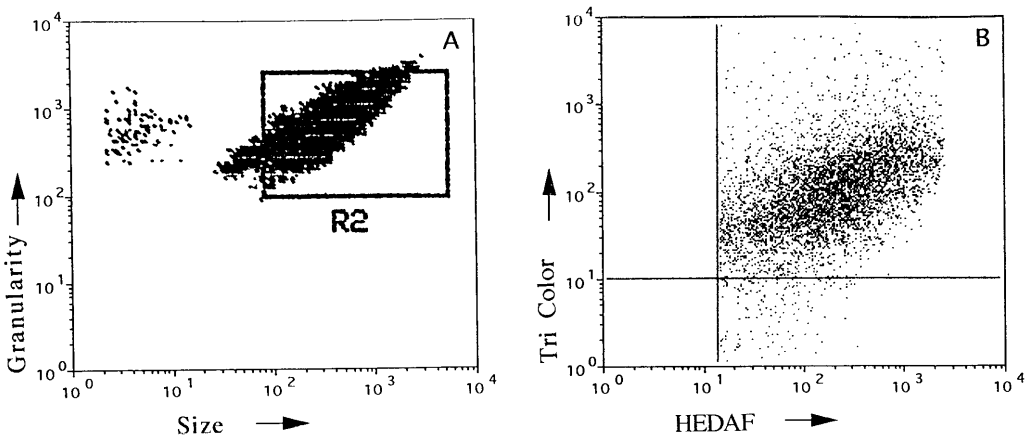

FIG. 3. Flow cytometric analysis of mycobacteria. (A) Two-dimensional computer contour plot showing the morphological distribution of cells defined by their size (forward angle light scatter) and granularity (side scatter). (B) Two-dimensional contour plot showing the distribution of HEDAF and Tri Color double-labeled cells.

CD4α) used as reference, a large number of cells showed high fluorescence intensities when specific MAbs were used. These intensities were judged significant when their values were four times above that obtained for the nonspecific binding. Furthermore, the different MAbs did not equally react with the various mycobacterial strains. For instance, the MAbs raised against the LOS-II of the Canetti strain of *M. tuberculosis* (9) strongly reacted with the Canetti-like strain, but not with the other mycobacterial strains tested (Table 2). This result is in agreement with the specific isolation and chemical characterization of this glycolipid from the Canetti strain and related strains (23), but not from the other strains of the tubercle bacillus (12, 22).

The PGL-tb-1 MAb gave significant reactions with the two other strains of *M. tuberculosis*, but not with *M. smegmatis* (Table 2). Because chemical analyses, conducted with the lipid extracts of the two positive strains, failed to detect PGL-tb-1, the positive reaction was interpreted as indicative of the presence of common yet unidentified epitopes on the cell surface of the strains.

The anti-ATA MAb reacted weakly with all strains, except strain H37Rv of *M. tuberculosis* (Table 2). This result is in accordance with a previous report concerning the failure to detect DMT among the surface-exposed lipids of these strains (31). The presence of DAT, a glycolipid that is also recognized by the MAb (Fig. 1), on the cell surface of strain H37Rv (31) may explain the relatively high fluorescence intensity of that strain.

Distribution of the ubiquitous mannosyl and arabinan epitopes on the cell surfaces of mycobacteria. The anti-arabinan MAb represented an interesting tool for comparison of the extent of exposure of the arabinan segments on the cell surfaces of various mycobacteria. In this respect, it has been shown that AMs and LAMs of slowly growing mycobacterial species were extensively capped with mannosyl residues at their nonreducing termini (7, 21, 24, 29, 32, 36), whereas those of rapidly growing species, including *M. smegmatis*, were not (6, 21, 24). Thus, one would expect that the anti-arabinan MAb reacts more with the cell surface components of slowly growing species than with those of the rapidly growing species examined. Surprisingly, as shown in Table 2, the MAb reacted equally well with the cell surface of *M. tuberculosis*, which is endowed with mannosyl-capped AMs, and with that of *M. smegmatis*, whose surface-exposed AMs are not capped with mannosyl residues. These data show that arabinan epitopes are exposed on the cell surfaces of all of the strains examined and are in agreement with immunocytochemical data obtained with *M. tuberculosis* (31). Furthermore, when exponentially pellicle-grown *M. tuberculosis* cells were shaken overnight in a fresh D-arabinose-containing culture medium, this resulted in the declumping of bacteria (2). Because the addition of either glucose or mannose did not lead to a similar observation, the result presented above strongly suggests that D-arabinose is involved in cell aggregation, thereby implying the exposure of the only surface compounds that contain the sugar (i.e., AMs), as previously firmly established (29, 30). It is worth noting that the three strains of *M. tuberculosis* examined differ in their abilities to react with the MAb (Table 2). This observation may be related to their different contents in surface-exposed AMs, and the presence of more arabinan epitopes on the cell surface of H37Ra than on that of the twin H37Rv strain (Table 2) is in

TABLE 2. Mean fluorescence intensities of mycobacterial strains reacting with various MAbs as determined by cytometry

Species	Fluorescence intensity (arbitrary units) with MAb[a]:					
	Anti-CD4α	Anti-arabinan	Anti-t-Man	Anti-ATA	Anti-PGL-tb-1	Anti-LOS-II
M. tuberculosis						
Canetti	140	1,830	4,160	480	5,170	1,760
H37Ra	170	2,640	5,340	400	1,400	100
H37Rv	210	900	3,400	1,070	1,630	30
M. smegmatis	200	1,571	3,900	450	60	191

[a] Fluorescence intensities are judged significant when their values are four times above that obtained for nonspecific binding (anti-CD4α).

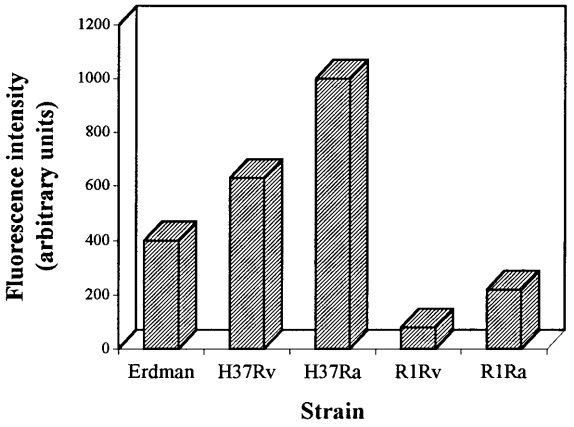

FIG. 4. Mean fluorescence intensities (arbitrary units) of different tubercle bacilli reacting with the anti-arabinan MAb by flow cytometry.

agreement with the relative content in surface-exposed AMs of the strains (29).

The anti-t-Man MAb reacted strongly with the surface of all of the strains examined (Table 2). It is worth noting that the fluorescence intensities obtained with the nonpathogenic species, *M. smegmatis*, were in the same range of magnitude of that obtained with the different strains of the tubercle bacillus. Because the AMs of the former species are not capped with mannosyl residues (24), the observed binding of the anti-t-Man MAb to the cell components obviously does not involve the oligomannosyl segments of the mannosyl-capped AMs. It has to be recalled that other mannoconjugates, including PIMs, are present on the cell surfaces of mycobacteria (29, 31). In the case of the strains of *M. tuberculosis*, however, the recognition of the mannosyl caps of AMs by the anti-t-Man MAb could not be excluded, especially because lipids represent only a very small portion of the surface-exposed material of the tubercle bacillus (30, 31). To test this hypothesis, more strains of the tubercle bacillus that differ one from the other in the amount and percentage of mannosyl capping of their surface-exposed AMs (29) and in their degrees of virulence were compared (Fig. 4). Examination of the data showed marked differences between the fluorescence intensities obtained with the various strains.

On the basis of the amounts of surface-exposed AMs and the percentage of mannosyl capping of these compounds (29), it is possible to predict for the various strains the relative amounts of terminal mannosyl residues that will react with the anti-t-Man MAb; this is easily obtained by multiplying the two parameters. Interestingly, the predicted reactivities of the five strains examined perfectly match the relative fluorescence intensities that result from the binding of the anti-t-Man MAb to the bacterial cells (Fig. 4). These data clearly suggest that the oligomannosyl residues which cap the AMs of the tubercle bacillus are primarily responsible for the binding of the MAb to the cell surface of *M. tuberculosis*. Although there was no correlation between the reactivities of the various strains with the anti-t-Man MAb and their degrees of virulence, it is worth noting that the avirulent strains R1Ra and H37Ra reacted more with the MAb than did the corresponding twin attenuated strain R1Rv and virulent strain H37Rv. Assuming that *M. tuberculosis* possesses several virulent factors, the presence of smaller amounts of surface-exposed mannoconjugates may be correlated to the virulence of the related pairs of strains R1RA/R1Rv and H37Ra/H37Rv. Further studies are necessary in order to test this hypothesis.

In conclusion, the flow cytometric method, as applied to mycobacteria, possesses several advantages over other methods, among which is the rapid and sensitive detection of surface antigens. Compared with the mechanical treatment of cells with glass beads, a method previously used for the identification of surface-exposed compounds (30, 31), it is worth noting that the analysis of mycobacteria by flow cytometric technique is much more sensitive and informative. The latter technique requires only very small amounts of cells and gives both qualitative (the accessibility of defined epitopes of the molecules) and quantitative information, after being compared with suitable references. Compared with immunocytochemistry, a technique commonly used for observing gold-labeled antibodies on negatively stained cells and thin sections, the cytometric method described herein is less time-consuming, because several strains may be comparatively analyzed in the same experiment. Furthermore, the quantification of surface-exposed antigens by the flow cytometric method is easier and statistically more reliable than the immunocytochemical analysis. Both techniques, however, require the use of antibodies, thus implying both the immunoreactivity of the substances to be analyzed and the availability of specific reagents. For the location of nonantigenic lipids, such as triacyl glycerol and dimycocerosate of phthiocerol, the isolation and chemical characterization of the compounds remain necessary.

With the availability of well-defined antibodies raised against an increasing number of defined mycobacterial antigens, it would be of interest to examine the physical arrangement and the amounts of various cell surface substances. Flow cytometric analysis may also be applied to mycobacteria which have been treated with drugs or other agents in order to gain insight into the arrangement of antigens in inner regions of the mycobacterial cell envelopes, hence contributing to our understanding of these fascinating organisms.

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REFERENCES

1. **Andersen, Å. B., and P. Brennan.** 1994. Proteins and antigens of *Mycobacterium tuberculosis*, p. 307–332. *In* B. R. Bloom (ed.), Tuberculosis: pathogenesis, protection, and control. American Society for Microbiology, Washington, D.C.
2. **Anton, V., P. Rougé, and M. Daffé.** 1996. Identification of the sugars involved in the mycobacterial cell aggregation. FEMS Microbiol. Lett. **44:**167–170.
3. **Bloom, B. R., and C. J. L. Murray.** 1992. Tuberculosis: commentary on a reemergent killer. Science **257:**1055–1064.
4. **Brennan, P. J., S. W. Hunter, M. McNeil, D. Chatterjee, and M. Daffe.** 1990. Reappraisal of the chemistry of mycobacterial cell walls, with a view to understanding the roles of individual entities in disease processes, p. 55–75. *In* E. M. Ayoub, G. H. Cassell, W. C. Branche, Jr., and T. J. Henry (ed.), Microbial determinants of virulence and host response. American Society for Microbiology, Washington, D.C.
5. **Chaisson, R.** 1993. Mycobacterial infections and HIV. Curr. Opin. Infect. Dis. **6:**237–243.
6. **Chatterjee, D., C. Bozic, M. McNeil, and P. J. Brennan.** 1991. Structural features of the arabinan component of the lipoarabinomannan of *Mycobacterium tuberculosis*. J. Biol. Chem. **266:**9652–9660.
7. **Chatterjee, D., K. Lowell, B. Rivoire, M. R. McNeil, and P. J. Brennan.** 1992. Lipoarabinomannan of *Mycobacterium tuberculosis*: capping with mannosyl residues in some strains. J. Biol. Chem. **267:**6234–6239.
8. **Cho, S.-N., J.-S. Shin, M. Daffe, Y. Chong, S.-K. Kim, and J.-D. Kim.** 1992. Production of monoclonal antibody to phenolic glycolipid of *Mycobacterium tuberculosis* and its use in the detection of the antigen in clinical isolates. J. Clin. Microbiol. **30:**3065–3069.
9. **Cho, S.-N., J. S. Shin, M. Daffé, Y. Chong, S. K. Kim, and J. D. Kim.** Unpublished results.

10. **Daffé, M., P. J. Brennan, and M. McNeil.** 1993. Major structural features of the cell wall arabinogalactans of *Mycobacterium*, *Rhodococcus*, and *Nocardia* spp. Carbohydr. Res. **249:**383–398.
11. **Daffé, M., C. Lacave, M. A. Lanéelle, and G. Lanéelle.** 1987. Structure of the major triglycosyl phenol-phthiocerol of *Mycobacterium tuberculosis* (strain Canetti). Eur. J. Biochem. **167:**155–160.
12. **Daffé, M., M. R. McNeil, and P. J. Brennan.** 1991. Novel type-specific lipooligosaccharides from *Mycobacterium tuberculosis*. Biochemistry **30:**378–388.
13. **Draper, P.** 1982. The anatomy of mycobacteria, p. 9–52. *In* C. C. Ratledge and J. L. Stanford (ed.), The biology of the mycobacteria, vol. 1. Academic Press, London.
14. **Draper, P.** 1991. The structure of the mycobacterial cell envelope is not yet understood. Res. Microbiol. **142:**420–422.
15. **Draper, P., and R. J. W. Rees.** 1970. Electron transparent zone of mycobacteria may be a defence mechanism. Nature (London) **228:**860–861.
16. **Fournié, J.-J., R. J. Mullins, and A. Basten.** 1991. Isolation and structural characteristics of a monoclonal antibody-defined cross-reactive phospholipid antigen from *Mycobacterium tuberculosis* and *Mycobacterium leprae*. J. Biol. Chem. **266:**1211–1219.
17. **Fournié, J.-J., M. Rivière, and G. Puzo.** 1987. Structural elucidation of the major phenolic glycolipid from *Mycobacterium kansasii*. Evidence for a tetrasaccharide structure of the oligosaccharide moiety. J. Biol. Chem. **262:** 3174–3179.
18. **Gaylord, H., P. J. Brennan, D. B. Young, and T. M. Buchanan.** 1987. Most *Mycobacterium leprae* carbohydrate-reactive monoclonal antibodies are directed to lipoarabinomannan. Infect. Immun. **55:**2860–2863.
19. **Hanks, J. H.** 1961. Significance of capsular components of *Mycobacterium leprae* and other mycobacteria. Int. J. Lepr. **29:**74–83.
20. **Hunter, S. W., and P. J. Brennan.** 1990. Evidence for the presence of a phosphatidylinositol anchor on the lipoarabinomannan and lipomannan of *Mycobacterium tuberculosis*. J. Biol. Chem. **265:**9272–9279.
21. **Khoo, K.-H., A. Dell, H. R. Morris, P. J. Brennan, and D. Chatterjee.** 1995. Inositol phosphate capping of the nonreducing termini of lipoarabinomannan from rapidly growing strains of *Mycobacterium*. J. Biol. Chem. **270:** 12380–12389.
22. **Lemassu, A., M. A. Lanéelle, and M. Daffé.** 1991. Revised structure of a trehalose-containing immunoreactive glycolipid of *Mycobacterium tuberculosis*. FEMS Microbiol. Lett. **78:**171–176.
23. **Lemassu, A., V. V. Lévy-Frébault, M. A. Lanéelle, and M. Daffé.** 1992. Lack of correlation between colony morphology and lipooligosaccharide content in the *Mycobacterium tuberculosis* complex. J. Gen. Microbiol. **138:**1535–1541.
24. **Lemassu, A., A. Ortalo-Magné, F. Bardou, G. Silve, M. A. Lanéelle, and M. Daffé.** 1996. Extracellular and surface-exposed polysaccharides of non tuberculous mycobacteria. Microbiology **142:**1513–1520.
25. **McNeil, M. R., and P. J. Brennan.** 1991. Structure, function and biogenesis of the cell envelope of mycobacteria in relation to bacterial physiology, pathogenesis and drug resistance; some thoughts and possibilities arising from recent structural information. Res. Microbiol. **142:**451–463.
26. **McNeil, M. R., M. Daffé, and P. J. Brennan.** 1990. Evidence for the nature of the link between the arabinogalactan and peptidoglycan of mycobacterial cell walls. J. Biol. Chem. **265:**18200–18206.
27. **McNeil, M. R., M. Daffé, and P. J. Brennan.** 1991. Location of the mycolyl ester substituents in the cell walls of mycobacteria. J. Biol. Chem. **266:**13217–13223.
28. **Misaki, A., N. Seto, and I. Azuma.** 1974. Structure and immunological properties of D-arabino-D-galactans isolated from cell walls of *Mycobacterium* species. J. Biochem. **76:**15–27.
29. **Ortalo-Magné, A., A. B. Andersen, and M. Daffé.** 1996. The outermost capsular arabinomannans and other mannoconjugates of virulent and avirulent tubercle bacilli. Microbiology **142:**927–935.
30. **Ortalo-Magné, A., M. A. Dupont, A. Lemassu, A. B. Andersen, P. Gounon, and M. Daffé.** 1995. Molecular composition of the outermost capsular material of the tubercle bacillus. Microbiology **141:**1609–1620.
31. **Ortalo-Magné, A., A. Lemassu, M. A. Lanéelle, F. Bardou, G. Silve, P. Gounon, G. Marchal, and M. Daffé.** 1996. Identification of the surface-exposed lipids on the cell envelopes of *Mycobacterium tuberculosis* and other mycobacterial species. J. Bacteriol. **178:**456–461.
32. **Prinzis, S., D. Chatterjee, and P. J. Brennan.** 1993. Structure and antigenicity of lipoarabinomannan from *Mycobacterium bovis* BCG. J. Gen. Microbiol. **139:**2649–2658.
33. **Sauton, B.** 1912. Sur la nutrition minérale du bacille tuberculeux. C. R. Acad. Sci. Ser. III Sci. Vie **155:**860–863.
34. **Schlesinger, L. S.** 1993. Macrophage phagocytosis of virulent but not attenuated strains of *Mycobacterium tuberculosis* is mediated by mannose receptors in addition to complement receptors. J. Immunol. **150:**2920–2930.
35. **Venisse, A., J. M. Berjeaud, P. Chaurand, M. Gilleron, and G. Puzo.** 1993. Structural features of lipoarabinomannan from *Mycobacterium bovis* BCG. J. Biol. Chem. **268:**12401–12411.
36. **Youmans, G. P.** 1979. Virulence of mycobacteria, p. 194–201. *In* G. P. Youmans (ed.), Tuberculosis. W. B. Saunders Co., Philadelphia.