Characterization of the epitope of anti-lipoarabinomannan antibodies as the terminal hexaarabinofuranosyl motif of mycobacterial arabinans

Devinder Kaur, Todd L. Lowary, Varalakshmi D. Vissa, Dean C. Crick and Patrick J. Brennan

mAb CS-35 is representative of a large group of antibodies with similar binding specificities that were generated against the Mycobacterium leprae lipopolysaccharide, lipoarabinomannan (LAM), and which cross-reacted extensively with LAMs from Mycobacterium tuberculosis and other mycobacteria. That this antibody also cross-reacts with the arabinogalactan (AG) of the mycobacterial cell wall, suggesting that it recognizes a common arabinofuranosyl (Araf)-containing sequence in AG and LAM, is demonstrated. The antibody reacted more avidly with ‘AraLAM’ (LAM with naked Araf termini) compared to ‘ManLAM’ (in which many Araf termini are capped with mannose residues) and mycolylarabinogalactan–peptidoglycan complex (in which the terminal Araf units are substituted with mycolic acids). Neither did the antibody bind to AG from emb knock-out mutants deficient in the branched hexa-Araf termini of AG. These results indicate that the terminal Araf residues of mycobacterial arabinan are essential for binding. Competitive ELISA using synthetic oligosaccharides showed that the branched hexa-Araf methyl glycoside \( [\beta-D-\text{Araf}\-(1\rightarrow2)-\alpha-D-\text{Araf}\-(1\rightarrow5)-\alpha-D-\text{Araf}\-(1\rightarrow5)-\alpha-D-\text{Araf}\-OCH_3] \) was the best competitor among those tested. The related linear methyl glycoside, \( \beta-D-\text{Araf}\-(1\rightarrow2)-\alpha-D-\text{Araf}\-(1\rightarrow5)-\alpha-D-\text{Araf}\-OCH_3 \), representing one linear segment of the branched hexa-Araf, was less effective and the other linear tetrasaccharide, \( \beta-D-\text{Araf}\-(1\rightarrow2)-\alpha-D-\text{Araf}\-(1\rightarrow3)-\alpha-D-\text{Araf}\-(1\rightarrow5)-\alpha-D-\text{Araf}\-OCH_3 \), was ineffective. The combined results suggest that the minimal epitope recognized by antibody CS-35 encompasses the \( \beta-D-\text{Araf}\-(1\rightarrow2)-\alpha-D-\text{Araf}\-(1\rightarrow5)-\alpha-D-\text{Araf}\-(1\rightarrow5)-\alpha-D-\text{Araf} \) within the branched hexa-Araf motif of mycobacterial arabinans, whether present in LAM or AG.

Keywords: M. tuberculosis, LAM, monoclonal antibodies, epitope definition, hexa-Araf motif

INTRODUCTION

The lipoarabinomannans (LAMs) of Mycobacterium species in their various forms have been implicated in a wide variety of important biological functions such as a generalized inhibition of T-cell function (Kaplan et al., 1987; Moreno et al., 1988; Molloy et al., 1990), induction of IL-12 (Yoshida & Koide, 1997) and evocation of CD-1 restricted T-cell responses (Sieling et al., 1995). In many of these functions, the Araf residues that are part of the non-reducing end unit of the molecule have been implicated, particularly in binding to various receptors. It had previously been shown that arabinosyl residues are responsible for the antigenicity of arabinogalactan (AG) and that serological activity resides largely in a fraction containing 2-linked arabinosyl residues (Misaki et al., 1974; Kotani et al., 1971). Moreover, it has long been assumed that the arabinans of LAM and AG are major B-cell immunogens, since, in...
Fig. 1. For legend see opposite.
very early work, it was shown that the decarboxylated forms of LAM, i.e. arabinomannan, and also arabino-galactan lost their antigenicity after treatment with arabinases (Misaki et al., 1977). Thus it was thought that part of or the entire non-reducing terminal motif is the major humoral immunological epitope of AG and of whole mycobacteria.

mAb CS-35 was raised against M. leprae LAM in this laboratory. This antibody has been used as a reference antibody for the characterization of mAbs against LAM and to study a variety of cellular functions such as the role of LAM in the interaction of mycobacteria with macrophages (Hamasur et al., 1999; Means et al., 1999; Schlesinger et al., 1994, 1996). However, the epitope structures recognized by this antibody have not been identified. The internal segments of arabinans, as they appear in both LAM and AG, consist of linear 5-linked α-D-Araf residues and some branched 3,5-linked α-D-Araf units substituted with 5-linked α-D-Araf residues at both branched positions (Fig. 1). The non-reducing terminal regions of the arabinans also contain 3,5-linked α-D-Araf residues substituted at both branched positions with the disaccharide β-D-Araf-(1→2)-α-D-Araf (Daffe et al., 1990). In the present study, several lines of evidence indicate that structural features within the terminal branched hexa-Araf arrangement constitute the epitope of mAb CS-35 and possibly many like antibodies.

**METHODS**

Reference materials. Anti-LAM mAb CS-35 (IgG3) (Hunter et al., 1986), AraLAM (LAM with naked Araf termini) obtained from a rapidly growing strain of mycobacteria (Chatterjee et al., 1991), ManLAM (LAM in which most Araf termini are capped with mannose residues) and lipomannan (LM) from *Mycobacterium smegmatis* (Chatterjee et al., 1992a) were kindly provided by Dr John Belisle through NIH, NIAID Contract NO1-AI-75320, ‘Tuberculosis Research Materials and Vaccine Testing’.

**Electrophoresis and Western blotting.** Purified LAM and LM preparations (5 μg) were applied to a Tricine 10–20% gel (Invitrogen) and electrophoresis was carried out at a constant 125 V for approximately 90 min. A sample of prestained molecular mass standards was also run on the same gel. After electrophoresis, gels were transblotted to nitrocellulose in Tris/glycine/methanol buffer under 56 V constant voltage. The nitrocellulose membranes were blocked in PBS containing 0.025% Tween-80 (PBST) and 1% BSA. CS-35 (diluted 1:1000) in PBST was added and incubated overnight at room temperature. The nitrocellulose membranes were then washed three times with PBST and incubated with anti-mouse IgG alkaline phosphatase conjugated antibodies (Sigma) for 1 h at room temperature. Bound antibodies were detected with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (p-nitro blue tetrazolium chloride (NBT) substrate (Sigma).

**ELISA.** Indirect ELISA, which measures binding of antibody to the immobilized polysaccharide-containing antigens, and competitive ELISA, which measures the ability of synthetic oligosaccharides to inhibit binding of antibody to immobilized AG, were carried out as previously described (Brito et al., 1985) with some modifications. Polysaccharide solutions (50 μl) were prepared in PBS pH 7.8 and applied to the wells of a flat bottom microtitre plate. The polysaccharides were immobilized on the untreated microtitre plates by incubating at 4 °C overnight. Non-specific antibody-binding sites were blocked by incubation for 1 h with 1% (w/v) ovalbumin and 0.05% Tween-80 in PBS (blocking buffer). mAb CS-35 (100 μl) diluted 1:1000 was added to the wells and allowed to incubate for 1 h. The wells were then washed with PBS containing 0.05% Tween-80 (washing buffer) (3 x 200 μl). Plates were incubated for 1 h with 100 μl antimouse IgG–alkaline phosphatase conjugate (Sigma) diluted 1:2000 in washing buffer and washed with PBS (3 x 200 μl). The alkaline phosphatase activity was measured using p-nitrophenyl phosphate as substrate (Kirkegard and Perry Laboratories). The liberated p-nitrophenol was quantified by measuring the absorbance at 405 nm.

**Competitive ELISA.** The synthesis of a range of oligosaccharides reflective of the termini of LAM and AG has been described (D’Souza & Lowary, 2000; Yin et al., 2002). These were prepared as the methyl glycosides. For assessment of their role in antibody binding, competitive ELISA was conducted. ELISA was carried out as described above except that 100 μl CS-35 (diluted 1:1000) plus varying concentrations of competitor solutions in blocking buffer were mixed and incubated at room temperature for 30 min prior to applying to the wells of the microtitre plate coated with AG or LAM. Bound CS-35 was detected as described above. The IC₅₀ is defined as the concentration of an oligosaccharide required to achieve 50% inhibition of the binding of mAb CS-35 to the immobilized AG. Values for controls with no competitor were taken as 0% inhibition of antibody binding and values from controls with no antibody represented 100% inhibition of binding.

**Preparation of AG and LAM from Mycobacterium smegmatis mc²155 and M. tuberculosis H37Rv.** M. smegmatis cells were grown to mid-exponential phase, harvested by centrifugation, washed with saline, lyophilized and extracted three times with CHCl₃-CH₂OH (2:1, v/v) at 50 °C for 2 h to delipidate the cells. The lipid extracts were subject to a partitioning step (Mikusova et al., 1995). LAM and LM were removed from the delipidated cells by repeated refluxing in 50% aqueous ethanol. The ethanol extracts were combined with the aqueous phase from the lipid extracts, evaporated to dryness, and partitioned between hot phenol and water, resulting in partially purified preparations of LAM mixed with LM. The aqueous layer containing the majority of cellular LAM and LM was freeze-dried and used for SDS-PAGE. The final insoluble material, which contained mycolylarabinogalactan-peptidoglycan covalent complex (mAGP) was further extracted with 2% SDS in PBS to remove proteins (Hirschfeld et al., 1990).

In the case of *M. tuberculosis*, after delipidation, cells were disrupted mechanically using a French pressure cell (American

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**Fig. 1.** Structure of the arabinan component of mycobacterial AG and LAM. The terminal hexa-Araf is similar in AG and AraLAM, whereas the terminal linear Araf segments are confined to LAM. In the case of mAGP, mycolic acids are attached to about two-thirds of the terminal hexa-Araf of AG. About 70% of these residues are mannosylated in the case of ManLAM. The polysaccharide LM consists essentially of the mannan core and phosphatidylinositol portion of LAM.
Instrument Company) at a pressure of 1000 p.s.i. (6.9 MPa). The suspension after cell breakage was centrifuged at 27000 g for 30 min at 4°C and the pellet was treated with SDS. SDS was removed by sequential washing with PBS, water, and finally with acetone. The insoluble mAGP was then treated with 1 M NaOH for 16 h at 80°C, followed by neutralization and dialysis to prepare the soluble arabino-galactan. The freeze-dried material was used to prepare alditol acetates for GC-MS analysis as previously described (Frehel & Leduc, 1987). The AG from embA, embB and embC ‘knock-out’ mutants of M. smegmatis (Escuyer et al., 2001) were prepared as described above.

RESULTS
Interaction of CS-35 with AG, LAM, LM and mAGP of mycobacteria

The obvious inference from the extensive body of work (Gaylord et al., 1987; Prinzis et al., 1993) demonstrating the reactivity of mAb CS-35 and like antibodies with the various forms of LAM is that it reacts with sugar arrangements at the non-reducing termini. The so-called branched hexa-Araf motif (Chatterjee et al., 1991) is the most common arrangement of Araf units at the non-reducing end of LAM (Fig. 1). Therefore, the reactivity of CS-35 with various forms of LAM (i.e. AraLAM and ManLAM) and LM was examined. The results of immunoblotting (Fig. 2) clearly implicated arabinan in that AraLAM and ManLAM showed comparable activity with CS-35 and LM did not react at all (Fig. 2). However, immunoblotting is a qualitative rather than a quantitative assay. Moreover, it should be noted that not all of the terminal Araf units of ManLAM are substituted with mannose residues. To obtain a quantitative measure that mAb CS-35, in fact, binds to Araf-containing termini, its comparative reactivity with the various forms of LAM (i.e. AraLAM and ManLAM) and AG was examined. The reasoning was that, if mAb reacts with both LAM and AG, then it must be binding to a shared arabinan component. CS-35 bound almost equally well to AG and AraLAM over a similar concentration range (Fig. 3). Moreover, the reactivity of CS-35 with ManLAM was less at lower concentrations and mAGP reacted very weakly. The terminal Araf units of these two products are extensively substituted with Manp or mycolic acid residues, respectively. mAb CS-35 did not react at all with LM. Clearly, the evidence of avid binding to both AraLAM and AG implicated the terminal Araf arrangements as the binding site of CS-35.

![Fig. 2](image1.png) Western blotting of LAM and LM preparations from mycobacteria. Lanes: 1, M. smegmatis AraLAM and LM (5 µg); 2, AraLAM (5 µg); 3, ManLAM (5 µg); 4, LM (5 µg). Blotting to nitrocellulose was performed at 56 V for 1 h. Western blots were conducted with mAb CS-35 (diluted 1:1000).

![Fig. 3](image2.png) Indirect ELISA measuring binding of monoclonal antibody CS-35 to various polysaccharides and lipopolysaccharides. Varying concentrations of polysaccharides were applied to Immunolon microtitre plates, and ELISA was performed as described in Methods. ●, AraLAM; ○, ManLAM; ▽, AG; ▽, LM.

![Fig. 4](image3.png) Reactivity of mAb CS-35 to AG isolated from embA, embB and embC knock-out mutants of M. smegmatis. Indirect ELISA was performed as described in Methods. ●, Wild-type AG; ○, embA; ▽, embB; ▽, embC; ■, embB (complemented).

![Fig. 5](image4.png)
These combined results indicate that the non-reducing Araf termini shared by AG and LAM are essential for binding to mAb CS-35.

**Interaction of CS-35 with embA, embB and embC knock-out mutants**

Ethambutol (EMB) specifically inhibits the synthesis of arabinan, both the AG and LAM versions (Takayama & Kilburn, 1989; Deng et al., 1995; Mikusova et al., 1995), and the genetic basis of EMB resistance has been traced to the *emb* operon containing the *embA, B* and *C* genes, presumably encoding arabinosyltransferases or arabinan transporters (Belanger et al., 1996; Telenti et al., 1997). Recently we demonstrated that *embA* and *embB* knock-out mutants are defective in the structure of the arabinan component of AG, in that the branch disaccharide, β-D-Araf-(1→2)-α-D-Araf, emanating from the 3-position of the 3,5-linked α-D-Araf residue, is largely deleted, whereas the *embC* mutant is defective in the production of AraLAM, while its AG is apparently normal (Escuyer et al., 2001). The availability of AG and AraLAM from these three mutants provided an op-
opportunity to precisely define the structure of the mAb CS-35 epitope. YHF-2-1, the complete hexa-Araf branched structure, YHF-3, a tetrasaccharide with the linear structure $\beta\text{-D-Ara}(1\rightarrow2)\alpha\text{-D-Ara}(1\rightarrow5)\beta\text{-D-Ara}(1\rightarrow5)\alpha\text{-D-Ara-OCH}_3$ representing the hexa-Araf without one of its branches, and YHF-5 with the structure $\beta\text{-D-Ara}(1\rightarrow2)\alpha\text{-D-Ara}(1\rightarrow5)\alpha\text{-D-Ara-OCH}_3$ representing the other linear segment of the hexa-Araf (Fig. 6) were tested for their ability to compete for binding of mAb CS-35 to AG and AraLAM by competitive ELISA. By way of controls, it was shown that AraLAM was almost as effective as intact AG (IC$_{50}$ 10 µg ml$^{-1}$) in inhibiting binding of CS-35 to immobilized AG (Fig. 7). YHF-2-1 (IC$_{50}$ 100 µg ml$^{-1}$) and YHF-3 (IC$_{50}$ 775 µg ml$^{-1}$) were effective competitors for the binding of mAb CS-35 to AG (Table 1). The best competitor was YHF-2-1, representing the full hexa-Araf branched structure. Moreover, YHF-3, representing one of the linear forms of hexa-Araf, also was effective, although less so. However, YHF-5 showed no ability to compete for the mAb CS-35 binding site. The terminal hexa-Araf released by arabinase digestion of M. tuberculosis H37Rv AG and isolated by semipreparative HPLC (Khoo et al., 2001) also showed inhibitory effects (data not shown), whereas the diarabinoside, $\alpha\text{-D-Ara}(1\rightarrow5)\alpha\text{-D-Ara}_f$, did not inhibit binding of mAb CS-35 to AG. A range of related monosaccharides (d-arabinose, d-ribose, d-galactose and d-mannose) were all very poor inhibitors. These data provide further support that terminal arabinosyl residues are an essential part of the epitope recognized by mAb CS-35.

**DISCUSSION**

mAb CS-35 reacted equally well with AG and AraLAM, suggesting that the terminal arabinosyl motif, which is the common motif shared by both macromolecules, is the main epitope recognized by mAb CS-35 (Fig. 3). ManLAM and mAGP from M. tuberculosis H37Rv showed decreased binding affinity to CS-35 due to the fact that 70% of the arabinosyl residues are mannose.
capped in ManLAM (Chatterjee et al., 1992a) and two-thirds are mycolylated in mAGP (McNeil et al., 1991). These observations indicate that masking of terminal Ara residues directly influences the binding with antibody. Chatterjee et al. (1992b) have demonstrated that AraLAM was 100-fold more effective in inducing tumour necrosis factor secretion than ManLAM. The observation that embA and B mutants, which were shown to be missing the disaccharide unit normally found on the 3-arm of the characteristic terminal hexa-Ara motif (Escuyer et al., 2001), did not readily bind to mAb CS-35 supports the overall evidence (Fig. 4). Furthermore, AG isolated from embC complemented and complemented embB knockout mutants, which have been shown to contain normal AG, showed no reduction in affinity for the CS-35 mAb. AraLAM isolated from embA and B mutants also maintained its capacity to interact with the antibody (Fig. 5), whereas AraLAM from the embC mutant, which is defective in some unknown aspect of the synthesis of LAM, exhibited no reactivity with mAb CS-35.

The epitope on AG recognized by CS-35 was further characterized using a panel of synthetic oligosaccharides (D’Souza & Lowary, 2000). Since attempts to bind the oligosaccharides directly to insoluble supports (either nylon membranes or plastic microtitre plates) failed, the relative affinity of mAb CS-35 for the various oligosaccharides was investigated in competitive ligand-binding studies against AG and AraLAM. As a control, competitive ELISA was performed in which AG or AraLAM was used as the immobilized antigen and various saccharides were used to competitively inhibit the binding of CS-35 to immobilized antigen. Some of the inhibitors, as well as AG and AraLAM, competitively inhibited the binding of CS-35 to immobilized AG, albeit with different efficiencies. The most effective competitor was AG (Fig. 7). AraLAM was a little less effective, whereas YHF-2-1 and YHF-3 were approximately 10-fold and 100-fold less effective as competitors, respectively. YHF-5, at the highest concentrations tested, 500 and 1000 μg ml⁻¹, did not compete with AG for binding to CS-35.

Several lines of evidence indicate that, although the terminal arabinosyl residues are a dominant part of the epitope recognized by mAb CS-35, it is not the only structural feature in AG recognized by the monoclonal antibody. The ability of arabinose-containing oligosaccharides to compete for the CS-35 site increased over 1 order of magnitude as the size of the oligosaccharide increased. Such size dependence would not be exhibited if the antibodies were recognizing only the terminal arabinosyl residues. Two possible explanations are offered for the difference in the behaviour of polymeric AG and oligosaccharides in the competitive binding assays. First, the superior ability of intact AG compared with AG oligosaccharides to compete for the antibody-combining site could be a reflection of the antibody’s avidity for a multivalent antigen. Alternatively, mAb CS-35 may recognize a conformation that is favoured in polymeric AG but that is only infrequently adopted by AG oligosaccharides of the sizes tested in this report. Levy et al. (1991) have shown that the most energetically favoured conformation of oligomers is the one in which the backbone adopts a twisted conformation, which is not conducive to the formation of hydrogen bonds between the backbone chains.

The binding and ligand competition experiments may provide additional insight into the nature of the polysaccharide binding site on mAb CS-35. We propose that the binding site consists of a groove, into which the polysaccharide chain fits, with one or more pockets that accommodate the terminal arabinosyl residues. Groove- and pocket-type binding sites on antibodies have been previously proposed based on results from ligand-binding and molecular-modelling studies (Cisar et al., 1975; Glaudemans, 1987; Oomen et al., 1991). X-ray crystallographic evidence of a pocket-type site on a monoclonal antibody against the bacterial O-antigen oligosaccharides has been obtained (Cygler et al., 1991). We hypothesize that the oligosaccharide, YHF-2-1 (Fig. 6), with two terminal arabinosyl arrangements, interacts with both the pocket and groove, thereby interfering with the binding of polysaccharide.

The epitope recognized by CS-35 is different from the galactose epitopes recognized by human intelectin (Tsuji et al., 2001). Human intelectin is a secretory glycoprotein consisting of 295 amino acids and N-linked oligosaccharides. It has affinities to D-pentoses and D-galactofuranosyl residues in the presence of Ca²⁺ and recognizes the arabino-galactan of Nocardia containing D-galactofuranosyl residues. The fact that reactivity of mAb CS-35 with AG decreased upon removal of arabinosyl residues and binds to synthetic arabinose oligosaccharides indicates that CS-35 recognizes a different epitope than does human intelectin.

Interesting spatial and developmental patterns of arabinogalactan epitope localization have been observed in different plant tissues using several antiarabinogalactan antibodies (Knox et al., 1991; Kikuchi et al., 1993; Pennell et al., 1992). Interpretation of these localization patterns at the molecular level is hampered by the lack of information on the epitope recognized by the antibodies used in these studies. The type of epitope characterization reported here for mAb CS-35 will now allow molecular definition of antibody–antigen interaction and thereby help with the interpretation of the spatial arrangement of AG in the mycobacterial cell wall. This information should also enhance the value of these antibodies for the screening of cell-wall mutants of mycobacteria lacking AG and AraLAM motifs and the recognition of biosynthetic intermediates of cell-wall polysaccharides unique to mycobacteria.

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