[Frontiers in Bioscience 3, e149-163, August 5, 1998]

RELATIONSHIPS BETWEEN THE STRUCTURE AND THE ROLES OF LIPOARABINOMANNANS AND RELATED GLYCOCONJUGATES IN TUBERCULOSIS PATHOGENESIS

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Received 7/5/98 Accepted 8/3/98

TABLE OF CONTENTS

1. ABSTRACT

The mechanisms and the molecular basis of the mycobacteria virulence remain obscure. However, recent findings provide evidences that, among the glycoconjugates which compose the mycobacterial envelope, lipoglycans including lipoarabinomannan, lipomannan and phosphatidyl-myo-inositol mannosides, are involved in the major steps of the tuberculosis immunopathogenesis. These steps are the mycobacterial phagocytosis process and the macrophage activation via the regulation of the production and secretion of cytokines.

In this article, we examine recent observations about comparative structural models of the lipoglycans from the pathogenic Mycobacterium tuberculosis strain, and the vaccinal M. bovis BCG strain, and finally avirulent mycobacteria strains. We also consider the role of the lipoglycans in the mycobacterial phagocytosis process and in the regulation of the macrophage microbicidal activity.

2. INTRODUCTION

Mycobacterium tuberculosis is a facultative intracellular pathogen which resides and survives within the phagolysosome of alveolar macrophages. Multiplication of M. tuberculosis within the cell is the major step for the pathogenesis of the disease. In the host, the earliest interaction between M. tuberculosis and the alveolar macrophages is the Mycobacterium binding to the cell surface followed by its internalization. At the molecular level, this interaction is mediated by molecules which are exposed at the surface of both the mycobacterial envelope and the host cell membrane.

Recent studies emphasize the importance of the complement receptor (CR) in the phagocytosis of M. tuberculosis by human monocytes and alveolar macrophages. *In vitro* investigations on human monocyte derived macrophages show that binding and phagocytosis of M. tuberculosis H37Ra are comparable to those of the Erdman and H37Rv virulent M. tuberculosis strains. Phagocytosis of these three strains was significantly inhibited using a combination of monoclonal antibodies directed against CR1, CR3, and CR4 in the presence and absence of serum. Phagocytosis was enhanced in the presence of fresh serum. Similar approaches have demonstrated that phagocytosis by human alveolar macrophages are drastically inhibited by CR antibodies. In contrast to CR, it was recently established that the mannose receptor, expressed in the membrane of alveolar macrophages, selectively mediates the adherence of the M. tuberculosis virulent strains.

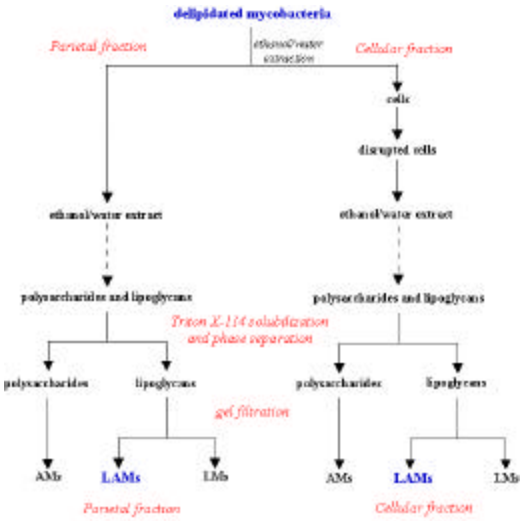

Figure 1. Purification scheme for LAMs.

A major challenge in mycobacteriology is the identification and the structural characterization of the envelope components which mediate the mycobacteria binding to the macrophage via the membrane receptors described above. The outer surface of M. tuberculosis and related mycobacteria contains large amount of complex glycoconjugates which are specific of the mycobacterial genus, and in some cases, of the species. Among these glycoconjugates, the lipoglycans including lipoarabinomannan (LAM) and lipomannan (LM) look as the prominent antigens since they modulate the macrophage functions.

As mentioned above, M. tuberculosis can multiply inside the alveolar macrophages. It was nevertheless observed that *in vitro* activation of murine macrophages by Interferon-gamma (IFN-gamma) and Tumor Necrosis Factor-alpha (TNF-alpha) promotes the destruction of intracellular bacteria. Again, it was established that LAM modulates the TNF-alpha secretion. Precisely LAMs from M. tuberculosis and M. bovis BCG induce much less TNF-alpha production from murine and human macrophage cell lines than LAMs from M. smegmatis and a fast growing mycobacterial strain do. Therefore a relation between virulence and the capacity of LAM to induce TNF-alpha production was propounded. This chapter summarizes our current knowledge of the mycobacterial lipoglycan structure and their immunological activities related to virulence and pathogenicity.

3. LAM PURIFICATION

The mycobacterial envelope is abundantly composed of mannoconjugates. The mannoconjugates can be classified in two groups, lipoglycans and polysaccharides. The lipoglycans are lipoarabinomannan (LAM), lipomannan (LM) and phosphatidylinositol mannosides (PIMs). Polysaccharides are restricted to arabinomannan (AM) and mannan. It is now clearly established that the early described D-arabinomannan exist in two forms, lipid-free, namely AM and acylated, namely LAM (1, 2). Likewise, mannan also exists in an acylated form, the LM.

In initial experiments, the mannoconjugates were extracted after vigorous alkali-treatments of the cells prior solvent extraction (3, 4). Consequently LAM and LM were deacylated leading to the formation of AM and mannan. In order to isolate the lipoglycans in their native form, the cells are extracted by solvents without any alkali treatment. The LAM purification from complex mixture containing structurally related molecules such as PIMs, LM and AM, still remains a critical task. Indeed, these molecules show a similar chromatographic behavior. Additionally, their separation is complicated by their ability to aggregate through hydrophobic or electrostatic interactions. Moreover, the difficulties encountered for LAM purification are heightened by their intrinsic molecular heterogeneity. This molecular heterogeneity was first illustrated by their behavior in SDS-PAGE, showing a broad band around 30 kDa (5) and more precisely defined by matrix-assisted laser desorption/ionization mass spectrometry analysis. The average molecular weight of LAM from M. bovis BCG was determined at 17 kDa and the heterogeneity estimated at 6 kDa (6).

Until now, LAM has been isolated following two kinds of extraction procedures. The main difference between these two methods is that LAM arises from ethanol/water extracts of either disrupted (7) or non-disrupted (5, 6) delipidated mycobacteria. Recently, a new method, resulting in a combination of the two previously described procedures, was developed (8). According to the extraction mode, two types of LAMs, the parietal and the cellular LAMs, were identified (8) (figure 1). The parietal LAM containing fraction results from ethanol/water extraction of delipidated mycobacteria. Afterwards, the resulting cells are disrupted, and then extracted again by ethanol/water, to give the fraction containing the cellular LAM. The routine separation steps for LAM purification from ethanol/water extracts employed in both procedures are similar, i.e. phenol/water biphasic wash to remove proteins, enzymatic degradation of the contaminants (glucan, nucleic acids, proteins), gel permeation to separate LAM and LM.

A critical purification step is the separation of glycans and lipoglycans. LAM, LM and AM have been tentatively separated by gel filtration and/or anion exchange chromatography. However, the aggregate formation between AM and, LAM and LM hinders their separation, even using detergents. In order to eliminate AM, prior gel filtration, the Triton X-114 phase separation technique was successfully applied (2). This non-ionic detergent favors the dissociation of amphipathic and hydrophilic molecules, and forms, at temperatures beyond the cloud point, a hydrophilic detergent-depleted phase and an amphipathic detergent-rich phase. AM is found in the former phase and LAM and LM in the latter one (figure 1). As expected, the

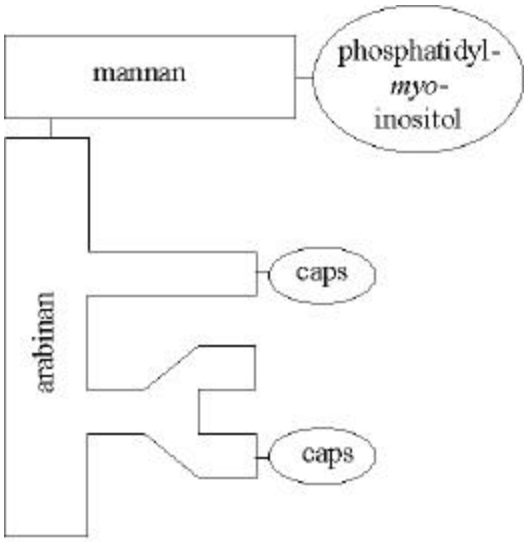

Figure 2. Schematic visualization of the tripartite structure of LAM.

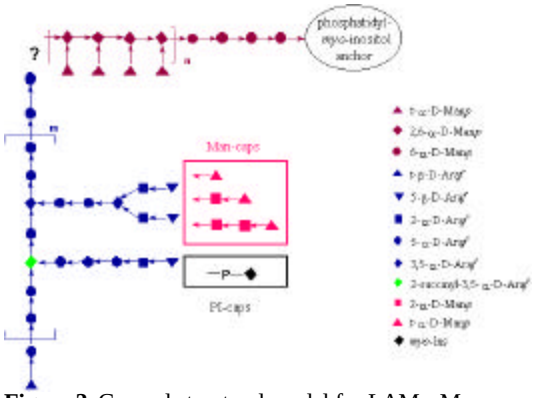

Figure 3. General structural model for LAMs. Man-caps characterize ManLAM and PI-caps PI-GAM and PI-LAM.

elimination of AM from the lipoglycan fraction, improves the separation of LAM and LM by gel permeation. A similar approach, allowing the extraction of lipoglycans by Triton X-114, was developed at the same time by an other group in New-Zealand (9). An other alternative, to separate lipoglycans and glycans, is the use of hydrophobic interaction chromatography on octyl-Sepharose. This technique allows the retention of lipoglycans and the elution of hydrophilic compounds (10).

The purification protocol described in the figure 1, devoted to the obtention of the so-called parietal and cellular LAMs, was applied to M. bovis BCG cells (2). Parietal LAM represents only 8% of the total LAMs. The amount of parietal and cellular LMs counts as the half of the amount of the corresponding LAMs. So, parietal lipoglycans represent a minor quantity of the total lipoglycan fraction. Likewise, the parietal polysaccharides AMs are found in lower abundance compared to the cellular ones (half), but polysaccharides relatively to lipoglycans, are more abundant in the parietal fraction than in the cellular one.

The epithets, parietal and cellular, used to characterize the two fractions of LAMs do not necessarily reflect a difference in the LAM localization in the mycobacterial envelope. Indeed, the localization of LAMs in the mycobacterial envelope is not yet established. LAM is not covalently attached since it can be extracted by solvents. At present, two models are proposed. Rastogi *et al*. (11) hypothesize that LAM could be inserted, through their phosphatidyl-myo-inositol unit, in an outer leaflet composed by different lipids. Mc Neil and Brennan (12) propose that LAM could be anchored, still by their phosphatidyl-myo-inositol unit, in the plasma membrane. In a recent review, Chatterjee and Khoo (13) privileged the second hypothesis relying on the relatively strong conditions required for LAM release from mycobacteria. This supposition has to be moderated since parietal LAM, contrarily to the cellular one, is obtained by ethanol/water extract without cell disruption. It can therefore be speculated that the major amount of LAM, i.e. the cellular one, could be inserted into the plasma membrane, while a minor amount, i.e. the parietal one, could be localized near the surface. It is noteworthy that the two previously proposed models concerning LAM localization are speculations since they are based on any convincing experimental support. Indeed, antibodies against LAM cross-react with AM precluding LAM identification, and so AM and LAM respective localization by immunocytochemistry experiments.

4. LAM STRUCTURE

LAM is composed by three domains, the polysaccharidic core, the phosphatidyl-myo-inositol anchor and the capping motifs (figure 2).

4.1 Polysaccharidic core

The polysaccharidic core is composed by two homopolysaccharides, D-mannan and D-arabinan. The mannan structure was determined following the mannan release from LAM either by mild acidic hydrolysis (14), which cleaves preferentially arabinofuranosyl linkages, or after action of an endoarabinanase (15). Invariably, D-mannan consists of a highly branched structure with alpha-(1→6) linked mannopyranosyl backbone substituted at C-2 by single mannopyranosyl units (figure 3). The mannan size and the branching degree can vary depending on the species. For example, mannan core of parietal LAM from M. bovis BCG is constituted by 18 Manp, with a branching degree, 2,6-alpha-Manp/(6-alpha-Manp + 2,6-alpha-Manp) ratio, of approximately 67% (14). Nevertheless this value is lower than the one found for AM mannan, 75% (14). The mannan core of LAM from M. smegmatis is composed by 26 residues on average, but poorly branched, ratio 2,6-alpha-Manp/(6-alpha-Manp + 2,6-alpha-Manp) around 50% (16).

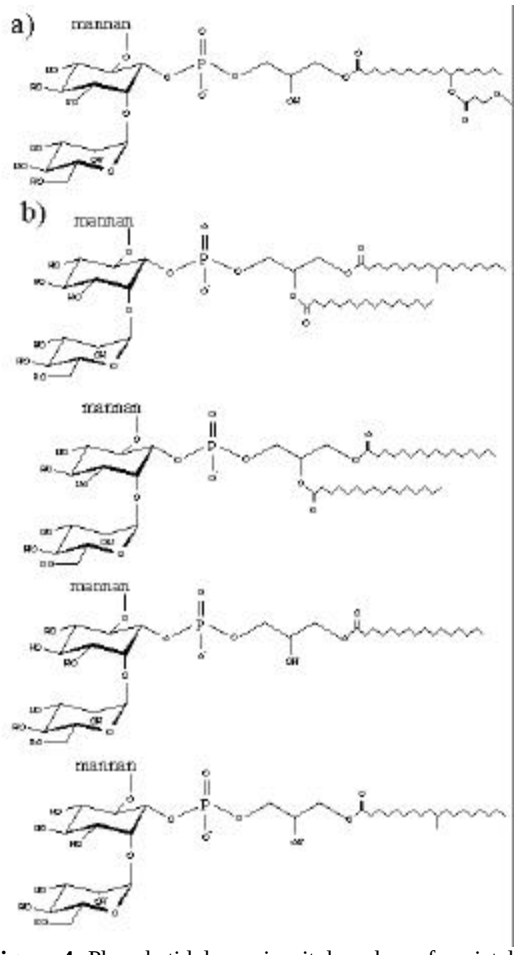

Figure 4. Phosphatidyl-*myo*-inositol anchor of parietal (a) and cellular (b) LAMs from *M. bovis* BCG. (R = fatty acid).

The arabinan domain consists of linear alpha-(1→5) linked arabinofuranosyl backbone punctuated by branching produced with 3,5-O-linked alpha-D-arabinofuranosyl residues (figure 3). The lateral chains consist in two arrangements, linear tetra-arabinofuranosides (Ara4): beta-D-Araf-(1→2)-alpha-D-Araf-(1→5)-alpha-D-Araf-(1→5)-alpha-D-Araf→ and bi-antennary hexa-arabinofuranosides (Ara6): [beta-D-Araf-(1→2)-alpha-D-Araf-(1-]2→3 and →5)-alpha-D-Araf-(1→5)-alpha-D-Araf→ (15, 17). In both cases, the non-reducing end is characterized by the following disaccharidic unit beta-D-Araf-(1→2)-alpha-D-Araf→. It can be noticed that only one 2-O-linked-alpha-D-Araf unit is present per side chain. Some beta-D-Araf units are substituted at C-5 by capping motifs (see capping section, 4.3). These general structural features are valid for LAM from slow growing mycobacteria, such as M. tuberculosis (15), as well as for LAM from fast growing mycobacteria, such as M. smegmatis or Mycobacterium sp. (16). Ara4 and Ara6 chains are in equal proportion in the LAM from M. smegmatis (16). Many authors have reported the presence of succinic and lactic groups on AM from M. smegmatis (18) and M. tuberculosis (19) and their occurrence has been postulated on LAM (5). But neither the existence of a covalent linkage nor their localization was defined. Using two-dimensional nuclear magnetic resonance spectroscopy, we demonstrated, in LAMs from four strains of M. bovis BCG (Pasteur, Glaxo, Copenhagen and Japanese) that one to four succinyl groups can esterify the 3,5-di-O-linked-alpha-D-Araf units at position 2 (8). These residues are labile and their presence on LAM seems dependent on mycobacterial culture. To date, the mode of linkage of arabinan domain on mannan core remains undetermined.

Only minor differences in the polysaccharidic core structure, such as mannan length or branching degree which do not modulate the LAM biological activities (see parts 5, 6 and 7), have been reported for LAM according to their mycobacterial origin. In summary, the structural model for polysaccharidic core (figure 3) seems ubiquitous for all the LAMs investigated.

4.2 Phosphatidyl-myo-inositol anchor

4.2.1 Structural features

The anchor structure is based on sn-glycero-3-phospho-(1-D-myo-inositol) unit with one alpha-D-mannopyranosyl unit linked at C-2 of the myo-inositol (figure 4). Additionally, the C-6 of myo-inositol unit is glycosylated by the mannan core (14, 20, 21). Variations in the anchor structure occur through the number, the localization and the nature of the fatty acids esterifying the anchor. The most abundant fatty acids, liberated after alkaline hydrolysis of unfractionated parietal and cellular LAMs (global fraction of LAMs from a given mycobacteria), are palmitic and tuberculostearic (10-methyl-octadecanoic) acids (20). Beside these fatty acids, stearic acid is found in small amount as well as traces of myristic, heptadecanoic, 10-methyl-heptadecanoic (10), 12-hydroxy-stearic and 12-hydroxy-tuberculostearic acids (2). Three fatty acid residues on average are found per molecule of LAM. Three sites of acylation are determined, i.e. positions 1 and 2 of the glycerol unit and position 6 of the Manp unit linked to C-2 of myo-inositol (7).

The fractionation of LAMs into parietal and cellular portions allows to separate LAM differing in their anchor structure. For example, the anchor of parietal LAM from M. smegmatis was found non-acylated (22). This LAM is called Phospho-Inositol-Glycero-Arabino-Mannan (PI-GAM) according to the cap structure (Phospho-Inositol, see capping section, 4.3.) and the absence of fatty acid residues (Glycero-Arabino-Mannan). This LAM which is devoid of fatty acids is not AM since it contains the anchor basic structure, i.e. sn-glycero-3-phospho-(1-D-myo-inositol) unit. The anchor of the cellular LAM from M. smegmatis is acylated, principally by the expected palmitic and tuberculostearic acids (Vercellone, unpublished data; 13). Hence this LAM is called PI-LAM. Likewise, differences in anchor structure have been described for the parietal and cellular LAMs from M. bovis BCG. Indeed, parietal LAM is esterified by only one fatty acid at the C-1 of the glycerol unit. The

fatty acid, assigned to the 12-O-(methoxypropionyl)-12-hydroxy-stearic acid, is unusual and found for the first time in the Mycobacterium genus (2) (figure 4a). Again, the cellular LAM contains palmitic, tuberculostearic acids and, in small amount, stearic acid. According to the analytical approach described above, four structures of acyl-glycerol are determined, those differ by the number and the nature of fatty acids (figure 4b). The four structures can be classified in diacylglycerols and monoacylglycerols. The diacylglycerols represent 72% and are esterified by palmitic acid at position 2 and tuberculostearic acid at position 1 (53%) or by two palmitic acids (19%). Monoacylcerols are less abundant (28%) and contain palmitic acid (22%) or tuberculostearic acid (6%) (2). Differences in anchor structure are also found for the parietal and cellular LAMs from M. tuberculosis strains (unpublished data).

4.2.2 Structural determination

The presence of a phosphatidyl-myo-inositol unit in LAM from M. tuberculosis was first proposed by Hunter *et al*. (5). This assignment was based on the characterization, by gas chromatography/mass spectrometry, of phospho-myo-inositol released by LAM alkalinolysis. However, it is known that the anchor phosphodiester linkage is alkali-stable. Actually, the investigated LAM was not from M. tuberculosis but from a contaminating strain assigned to a fast growing mycobacterial strain (16). Today, it is known that this LAM is capped by phosphoinositides (see capping section, 4.3). The phosphoinositide caps are also described in the LAM from M. smegmatis, where they are shown to be alkali-labile (22) (see capping section, 4.3). So it is likely that the phosphinositols obtained from the LAM alkalinolysis (5) characterize the cap motifs and not the anchor. Indeed, the same authors have unambigously demonstrated, by selective LAM radiolabeling (3H myo-inositol), the presence of both alkali-stable and alkali-labile phospho-inositol (20). As expected, the glycerol unit was only found after acidic hydrolysis in the alkali-stable fraction. The presence of palmitic and tuberculostearic fatty acids on one hand, and phospho-myo-inositol and glycerol on the other hand, led to propose the presence of a phosphatidyl-myo-inositol anchor (20). Additionally, by conventional methylation carbohydrate analysis, the myo-inositol unit appears substituted at positions 2 and 6 by Manp residues and at position 1 by a phosphate group (21). This analysis strategy requires chemical degradations of the anchor. So, we have intented a nuclear magnetic resonance approach, based on two-dimensional 1H-31P experiments applied on native LAM, which allows the structural characterization of the anchor. The nuclear magnetic resonance approach was first adjusted to the mannan core of parietal LAM from M. bovis BCG, and then successfully applied to deacylated LAM (14, 23), and finally to native parietal LAMs from M. smegmatis (22) and M. bovis BCG (2). This method allows to determine the anchor structure, i.e. myo-inositol substitution and glycerol acylation. Unfortunately it is unsuccessful to determine the anchor structure of the cellular LAM from M. bovis BCG. Indeed, the one dimensional 31P spectrum of cellular LAM from M. bovis BCG shows a broad unresolved signal due to LAM molecular aggregation in D2O. This phenomenon is explained by a higher number of fatty acids present in the cellular LAM than in the parietal one (2).

To determine the structure of the acyl residues borne by the glycerol, a new analytical procedure was employed. LAM is submitted to acetolysis allowing the cleavage between phosphate and glycerol but preserving acyl-glycerol residues. Acetolysates are analyzed by gas chromatography/electron impact-mass spectrometry and gas chromatography /chemical ionization-mass spectrometry (2). The use of a short capillary column permits the separation of glycerides by gas chromatography. Molecular mass determined from chemical ionization-mass spectrometry experiments and analysis of fragment ions from electron impact-mass spectrometry experiments resolve the nature and the position of fatty acids on glycerol.

4.3 Capping motifs

Two types of capping motifs have been identified, mannooligosaccharides for LAMs from the slow growing mycobacteria, M. tuberculosis, M. leprae, and M. bovis BCG, these LAMs have been called ManLAMs; and phosphoinositides for LAMs from the fast growing mycobacteria, M. smegmatis and Mycobacterium sp., these LAMs have been termed PI-GAM and PI-LAM.

4.3.1 Structural features

Mannooligosaccharide caps were first evidenced by Oashi *et al*. (24) suggesting that the arabinosyl side chains of AMs from M. tuberculosis Aoyoma B were terminated with mannosyl residues. Chatterjee *et al*. (25) have stated that capping motifs typifying ManLAMs from M. tuberculosis are a single Manp, a dimannoside (alpha-D-Manp-(1→2)-alpha-D-Manp) or a trimannoside (alpha-D-Manp-(1→2)-alpha-D-Manp-(1→2)-alpha-D-Manp) (figure 3). Dimannosyl units represent the major motifs (77%) of parietal and cellular LAMs from M. bovis BCG, while mannosyl (16%) and trimannosyl (7%) are less abundant (2). These motifs are present on both linear Ara4 and bi-antennary Ara6 side chains of ManLAM from M. tuberculosis (figure 3). On linear chains, the major motifs found are di- and trimannosides (Manp2Araf4 and Manp3Araf4), while on bi-antennary chains, all the combinations are present, i. e. from 1 to 6 Manp units (Manp1 to 6Araf6) (15). The Man-cap structures are identical for all the investigated ManLAMs, but differences occur in the capping extension. The capping degree corresponds to the ratio between the number of arabinan chains terminated by Manp units and the total number of arabinan chains (for the ratio value determination, see 4.3.2). The capping degree of ManLAM varies according to the mycobacterial species (table 1). On unfractionated parietal and cellular ManLAMs, ManLAMs from M. tuberculosis Erdman strain and M. bovis BCG are the most capped compared to the ManLAMs from M.

Table 1. Capping percentage of ManLAMs from different *Mycobacterium species*.

Capping percentage	M. tuberculosis			M. leprae[a]	M. bovis BCG		
	H37Ra[a]	H37Rv[a]	Erdman[b]		total fraction[c]	parietal[d]	cellular[d]
(1)	43	44	71	0	73	69	24
(2)	51	64	78	20	79	76	48

1. (2-*O*-linked Ara*f* - t-Ara*f*) to 2-*O*-linked Ara*f* ratio. 2. (3,5-di-*O*-linked Ara*f* - t-Ara*f*) to 3,5-di-*O*-linked Ara*f* ratio. [a] (16); [b] (25); [c] (26); [d] (2).

tuberculosis H37Ra and H37Rv strains. The capping of the ManLAM from M. leprae is very modest, and calculation of the capping degree lead to a value next to 0 (16) (table 1). Differences in the capping degree are also observed between the parietal and cellular ManLAMs from M. bovis BCG; the former ones being more capped than the latter ones.

Phosphoinositides are designated as capping motifs of LAM (previously called AraLAM) from the fast growing mycobacteria, Mycobacterium sp., initially considered as M. tuberculosis (16). Inositol phosphate is recognized as capping the non-reducing end of linear arabinan chains (Ara4) exclusively (figure 3). About 20% only of the arabinan side chains of LAM from the unidentified Mycobacterium sp. are found capped (16). Phosphoinositide caps are also described in the parietal LAM from M. smegmatis (PI-GAM) (22). Moreover, the precise structure of these caps is established, by two-dimensional 1H-31P nuclear magnetic resonance experiments, as (myo-inositol-1)-phosphate-(5-beta-D-Araf) (22) (figure 3). The presence of four phosphoinositide residues per molecule of PI-GAM is assigned to approximately 40 to 50% of capping. These caps are alkali-labile (22), but the molecular mechanism of alkalinolysis remains unclear.

4.3.2 Structural determination

2-O-linked-alpha-D-Manp units characterize mannooligosaccharide caps. Their identification by standard methylation analysis let to recognize the analyzed LAM as ManLAM (25). A non-destructive approach, based on two-dimensional heteronuclear 1H-13C nuclear magnetic resonance experiment, was developed for the characterization of ManLAM (6, 23). The ManLAMs are specified by two types of 2-O-linked Manp, one arising from the mannan core (2,6-di-O-linked Manp) and the other one from the mannooligosaccharide caps (2-O-linked Manp). The anomeric carbon signals of these two units have the same chemical shift but correlate with two different anomeric proton resonances, as evidenced in 1H-13C heteronuclear multiple quantum correlation experiment. These two anomeric proton resonances reveal the presence of the two types of 2-O-linked Manp characterizing ManLAM. Only one proton resonance corresponding to 2,6-di-O-linked Manp is observed when PI-GAM from M. smegmatis is analyzed, this typifies the previously called AraLAM characterized by the absence of mannooligosaccharide caps.

As noticed above, the capping degree allow to characterize different ManLAMs according to their mycobacterial origin, and also to differ between parietal and cellular ManLAMs. The capping degree is determined from methylation analysis data. Different calculations are employed. Khoo *et al*. (16) invoke the presence of one 2-Araf unit per arabinan side chain to compute the capping degree from the ratio (2-O-linked Araf - t-Araf) to 2-O-linked Araf (ratio 1). Concurrently, Nigou *et al*. (2) make use of the ratio (3,5-di-O-linked-Araf - t-Araf) to 3,5-di-O-linked-Araf (ratio 2). Unfortunately, as shown in table 1, these two types of calculations, applied on the same methylation data from a given ManLAM lead to different values. The drawback of this method is the determination of the relative abundance of the different monosaccharides composing ManLAM (3,5-di-O-linked-Araf, 5-O-linked Araf, 2-O-linked Araf, t-Araf, 2,6-di-O-linked Manp, 6-O-linked Manp, 2-O-linked Manp, t-Manp) from their corresponding alditol acetate. Indeed, ManLAM hydrolysis process probably discriminates between di-O-linked monosaccharides, mono-O-linked monosaccharides and terminal monosaccharides distorting the relative abundance of the different alditol acetate species. Ratio 2 provides higher capping degree values than ratio 1 but whatever the ratio used the largest and the poorest capped ManLAMs remain the same. Finally, as the method is based on permethylation by the Ciucanu and Kerek technique, it turns out to be inadequate for determining the capping degree of PI-GAM and PI-LAM. Indeed, phosphoinositide caps are alkali-labile and would be loss during the permethylation step which needs sodium hydroxide. This problem could be overcome using the Prehm methylation method (27).

As mentionned above, the Man-caps are composed by different mannooligosaccharides (mono-, di- and trimannosides). In order to identify and quantify these different motifs, an approach based on mild acidic hydrolysis of ManLAM (0.1 N HCl, 30 min at 110°C), tagging by aminopyrene trisulfonate followed by capillary electrophoresis analysis monitored by laser-induced fluorescence, was developed (2). The analysis can be performed in the microgram range of ManLAM and will be useful for the screening of ManLAM from mutant strains.

5. LIPOGLYCANS MODULATE CYTOKINE SECRETION

It is well-documented that mycobacteria trigger off the cytokine release from the host immune cells. For example, mycobacteria stimulate, *in vivo* and *in vitro*, the release of Tumor Necrosis Factor (TNF-alpha), Granulocyte Macrophage-Colony Stimulating

Factor (GM-CSF), Interleukin 1 (IL-1), IL-2, IL-6 and IL-10 from different types of phagocytic cells (28-35). Some of these cytokines are involved in the modulation of the macrophage activation, and, consequently, of the intramacrophagic mycobacterial survival. Indeed, production of TNF-alpha and GM-CSF is associated with growth inhibition or killing (36-42), whereas IL-1 and IL-6 can promote mycobacterial growth (38, 40). The stimulation of cytokine secretion by the host immune cells is modulated according to the mycobacteria virulence. Virulent species appear as weaker inducer of cytokines release such as IL-1alpha, IL-1beta, IL-6 and TNF-alpha (43, 44), than avirulent strains. This fact emphasizes the role of the cytokines in the immunopathogenesis of the tuberculosis.

Thus, a comprehensive understanding of the mycobacteria pathogenicity requires the exploration of the mycobaterial molecules implicated in the regulation of cytokine secretion. Indeed, in the past, protein antigens (45-49), protein-peptidoglycan-complex (30), trehalose dimycolate (50), sulfatides (51) were found to stimulate cytokine secretion from phagocytes. More recently, lipoglycans and particularly LAM, emerge as potent inducers of cytokine. This chapter summarizes the current knowledge of the cytokine induction by the mycobacterial lipoglycans.

5.1 Lipoglycans and TNF-alpha

TNF-alpha is a cytokine polypeptide with pleiotropic effects depending on its relative concentration and presence of other biological mediators (52). In the tuberculosis pathogenesis, the TNF-alpha is involved in the formation of the granuloma (53) which prevents the mycobacteria dissemination. In addition, alone or in synergy with Interferon-gamma (IFN-gamma), it stimulates the phagocyte bactericidal activity (54-56). In that way, TNF-alpha launches a protective immune response. However, the action of TNF-alpha may be to the detriment of the host, causing tissue damage (57-59), fever and weight loss.

Moreno *et al*. (60) were the first authors to show that purified LAM is a potent inducer of TNF-alpha in human and murine macrophages. A few years latter, it was observed (61-63) that the amount of TNF-alpha released differs according to the mycobacterial origin of the LAM. Indeed, LAMs from non virulent mycobacterial species, called AraLAM were found more potent than those of virulent species, namely ManLAM. This activity was established using peritoneal and bone-marrow-derived macrophages and LAMs from the M. tuberculosis Erdman strain and an unidentified fast growing mycobacterial species initially considered as H37Ra. To date, ManLAM has been isolated from all strains of the M. tuberculosis group (virulent strains: Erdman strain, H37Rv; attenuated strains : H37Ra, M. bovis BCG) and from M. leprae, while AraLAM has been purified from rapidly growing mycobacteria. The structural features of AraLAM involved in the TNF-alpha release have been investigated. It was established that alkaline treatment of AraLAM leads to a drastic decrease of TNF-alpha induction. A deduction of this result was that the fatty acids esterifying the glycerol moiety were involved in this process. This assumption was supported by the fact that all lipoglycans containing a phosphatidyl inositol anchor (AraLAM, LM and PIMs) are also able to trigger off cytokine induction by human monocyte (62). However, in murine macrophages (63) LM and PIMs induce low levels of TNF-alpha, comparable to those induced by ManLAM. More recently, the presence of alkaline labile groups different from the fatty acyl residues was ascertained in LAM from M. smegmatis. Indeed, we have shown that the parietal LAM from M. smegmatis, contains alkali phosphoinositide caps (22). Moreover, this parietal LAM, called PI-GAM, is devoid of fatty acid residues. It is nevertheless a potent inducer of TNF-alpha from THP-1 monocyte/macrophages human and J774E macrophage murine cell lines. In contrast, the cellular LAM from M. smegmatis, namely PI-LAM, characterized by the presence of fatty acid residues and phosphoinositide caps (unpublished data), is drastically less active than the parietal one. The PI-GAM activity is markedly diminished after its alkali-treatment underlying the major role of the phosphoinositide caps (22).

In summary, it can be noticed that avirulent mycobacterial strains contain LAMs which are able to activate the macrophage microbicidy via the stimulation of the TNF-alpha secretion. On the contrary, this activity is considerably lower for ManLAMs from virulent strains such as the M. tuberculosis strains, and BCG. These data, observed *in vitro* using monocyte, and human and murine cell lines, justify the likelihood of a correlation between LAM activity and mycobacteria intramacrophagic survival.

The LAM activity was also investigated using immature dendritic human cells which are specialized in the antigen capture and in the stimulation of T cells (64). The parietal ManLAM from BCG, contrarily to the cellular one, is able to stimulate TNF-alpha (2). As mentioned above, parietal and cellular ManLAMs differ in the lipid part of the phosphatidyl-myo-inositol anchor and the degree of the mannose capping. Two others studies (65, 66) have described similar TNF-alpha inducing capacity for ManLAM and AraLAM. In the first one (65), ManLAM from M. tuberculosis strain Erdman and AraLAM from a rapid growing Mycobacterium species have been tested on microglial cells, the resident macrophages of the brain. The second one (66) has used ManLAM from M. avium on resident and thioglycollate-elicited macrophages.

Taken together, all these data highlight that mechanisms of TNF-alpha induction by mycobacterial lipoglycans depend on both their structural features and also the type of immune target cells. It can be assumed that the cytokine induction level may be quite different according to the protein receptors expressed in the host cell membrane. Therefore, enlightenment on the LAM molecular target represents a major step in the full understanding of the molecular mechanism involved in the regulation of the TNF-alpha induction by LAM.

The mechanism of the regulation of TNF-alpha production by AraLAM was first investigated by Zhang *et al*. (67) and Brown and Taffet (68). The implication of the LPS receptor CD14 was demonstrated by the use of anti-CD14 monoclonal antibody that inhibits TNF-alpha release induced by AraLAM or LPS. However, large amounts of AraLAM or LPS elicit the secretion of TNF-alpha by mononuclear phagocytes despite the presence of anti-CD14 antibodies (67), suggesting the existence of an additional mechanism. The additional mechanism could involve another protein, distinct from CD14, at the cell surface of leukocytes which has been recently shown participating in LPS uptake (69, 70). Moreover, fibroblast cell lines transfected by CD14 and responsive to LPS, are not activated by AraLAM. So, in contrast to the LPS, the AraLAM recognition via CD14 would require an additional receptor which the expression seems to be restrict to the hemopoietic cells (70). Furthermore, the LPS induction of TNF-alpha transcription is clearly mediated in part by the transcription factor NF-kapaB (71-73). Indeed incubation of LPS with human and murine macrophages, results in an activation of this transcriptional factor. As expected, AraLAM shares this activity, while ManLAM from M. tuberculosis is a 5-fold less efficient stimulus (68). These experiments have been performed on murine bone marrow-derived macrophages, RAW264.7 murine macrophage-like cell lines. When using another murine macrophage-like cell line, the J774A cell line, ManLAM induces thereby a significant activation of NF-kapaB, similar to that of AraLAM. These data highlight once again the complexity of the molecular mechanisms involved in the regulation of the macrophage TNF-alpha production by the LAM.

More recently, an elegant work (74) revealed that ManLAM from Erdman strain of M. tuberculosis abrogates tyrosine phosphorylation of human monocyte proteins induced by phorbol myristate ester, LPS or IFN-gamma. LM and PIMs from M. tuberculosis H37Rv have the same action but to a lesser extent. Hence, this activity seems to depend, at least partially, on the polysaccharide frame of the lipoglycans. The attenuation of protein phosphorylation by ManLAM is due to the activation of phosphotyrosine phosphatase SHP-1, leading to a significant reduction of LPS-induced TNF-alpha production. Interestingly AraLAM from M. smegmatis does not share this functional activity with ManLAM. In contrast, it acts like LPS in triggering off tyrosine phosphorylation. Unfortunately, in this study, the inhibition of AraLAM-induced TNF-alpha production by ManLAM was not investigated. However, we have shown that an incubation of THP-1 cells with both PI-GAM and ManLAM from M. bovis BCG in different ratios (from 1:1 to 1:10 PI-GAM:ManLAM) does not lead to inhibition of PI-GAM-induced TNF-alpha release (unpublished results). Conversely for 1:10 ratio a slight but significant increase of TNF-alpha level was observed. Moreover, Dahl *et al*. (75) have shown that pretreatment of primary human monocytes with M. tuberculosis ManLAM does not inhibit LPS- and AraLAM-TNF-alpha induction.

5.2 Lipoglycans and other cytokines

Beside TNF-alpha, lipoglycans from mycobacteria are also able to induce cytokines secreted by phagocytic cells, such as GM-CSF, IL-1alpha, IL-1beta, IL-6, IL-8, IL-10 and IL-12, but not those produced by lymphocytes (lymphotoxins, IFN-gamma, IL-2, IL-3 and IL-4 (62). Among these cytokines, some have pro-inflammatory actions (IL-1, IL-6, IL-8 and GM-CSF) whereas others are anti-inflammatory cytokines (IL-10, IL-12 and Transforming Growth Factor (TGF-beta)) (76).

AraLAM is described as a more potent inducer of pro-inflammatory cytokines than ManLAM. Indeed IL-1 induction has been reported in human monocytes incubated with AraLAM from a rapid growing mycobacterium species (62, 67, 75, 77). Zhang *et al*. (67) have provided evidences based on inhibition of IL-1beta release by anti-CD14 monoclonal antibodies, that AraLAM acts through the LPS receptor. IL-1 displays the biological properties of TNF-alpha (for a review see (76) and references therein). So, in the first steps of infection this cytokine may be protective (78), particularly by a chemotactic and proliferative action on lymphocytes T leading to the release of IFN-gamma (79). Moreover IL-1 could directly induce a granulomatous response (80). And it has been proved that IL-1beta induces IL-6 (81) and IL-8 (82, 83) production. While IL-8 seems to play a positive role in immuno-pathology in recruiting neutrophils (84) and T cells (85) and enhancing the intracellular killing of mycobacteria (86), IL-6 increases the intracellular and extracellular growth of M. avium (87) and down-regulates TNF receptors (88). AraLAM and related lipoglycans stimulate IL-6 secretion by polymorphonuclear monocytes (62, 75, 89) through the activation of the nuclear factors NF-IL-6 and NF-kapaB on the IL-6 gene (89). Concurrently, IL-8 induction by glycoconjugates from a rapid growing mycobacteria (90, 91) and from M. bovis BCG (2) could result in an indirect stimulation of target cells since neutralizing antibodies against TNF and/or IL-1alpha and -beta blocked 83% of the stimulation (90).

Peptide cytokines KC and JE, chemoattractants for neutrophils and monocytes respectively, are induced by AraLAM but not ManLAM (92, 93). However, differential modulation of LAM-induced chemoattractants by IFN has been observed (92, 93).

In contrast to the abundant literature about the lipoglycans-induced pro-inflammatory cytokines, only a few reports concern the regulation of anti-inflammatory cytokines by these lipoglycans. AraLAM was found to be a more efficient inducer of IL-10 (75) and IL-12 (94) than ManLAM, although for IL10, both LAMs are poor stimulators. In contrast both LAMs have a similar action on monocytes for the secretion of TGF-beta. It seems that IL-10 and TGF-beta could have a negative role in immune defense against mycobacteria since they diminish the microbicidal activity of macrophages (95-

98). IL-12 appears to trigger off a T cell dependent protection.

In conclusion, AraLAM and particularly PI-GAM upregulate the production of numerous phagocyte-secreted cytokines and particularly the ones which would possibly have a protective role against the mycobacterial infection.

6. LIPOGLYCANS MODULATE MACROPHAGE EFFECTOR FUNCTION

LAM is also a potent downregulator of several functions linked to the host cell mediated immunity (99). Ellner and Daniel (100) were the first to report that ArabinoMannan, purified from culture filtrates of M. tuberculosis, suppresses macrophage-dependent, antigen-induced lymphoproliferation. ManLAMs from M. leprae and M. tuberculosis were found to have the same effect (101-103) probably through inhibition of protein-antigen processing by antigen-presenting cells (101). ManLAM also blocks the IFN-gamma-mediated activation leading to an inhibition of stasis and cidal activities of macrophages (104-106). It has been reported that the unresponsiveness to IFN-gamma is not due to a defect in binding or in internalization of this cytokine by ManLAM-treated macrophages (105). In fact, ManLAM was found to act at the transduction signal and transcriptional activation levels by inhibiting the protein kinase C activity (106) and by blocking the IFN-gamma-inducible gene transcription (105, 106). The inhibition of interferon-gamma activation requires a pretreatment of the macrophages with ManLAM. In contrast, the microbicidal function of activated macrophages treated with ManLAM is not affected (105).

Several reports have shown that LAM could induce the production of reactive nitric intermediates (61, 107, 108) and the toxoplasmacidal activity by normal IFN-gamma-primed macrophages (61). Interestingly AraLAM could elicit NO2- release in both normal and primed macrophages, whereas ManLAM does it only in primed-macrophages (61). Moreover AraLAM is a more potent inducer than ManLAM (108).

A better understanding of the LAM activity on the regulation of the cytokine production by the phagocytes will required the characterization of the receptors implicated in the LAM uptake.

7. PROTEIN RECEPTORS OF THE LAM

Among receptors that have been described to interact with mycobacteria, three, CD14 (see above), the mannose receptor and the mannose binding protein, involve LAM and related lipoglycans. The mannose receptor participates in a non opsonin-mediated phagocytosis by binding and internalizing the glycoconjugate capped with mannose, fucose or N-acetyl glucosamine (for a review see (109)). Schlesinger (110) was the first to report that, in addition to complement receptors, M. tuberculosis binds to macrophage also via the mannose receptor. Demonstration of the mediated-mannose receptor binding was based on inhibition using mannan, mannose-bovine serum albumin, N-acetyl glucosamine-bovine serum albumin and antibodies directed against mannose receptor. Interestingly, while virulent and attenuated strains of M. tuberculosis (H37Rv, Erdman and H37Ra) bind the phagocyte via the complement receptors, only the virulent strains interact with the mannose receptor. Thus, for the first time was described a phagocyte receptor which discriminates between virulent and attenuated strains of M. tuberculosis, and therefore differentiates a pathway susceptible to have an influence on the intracellular fate. One year later, Schlesinger *et al*. (111) provide evidence, based on the use of microsphere coated with mycobacterial lipoglycans, that ManLAM is the mannose receptor ligand. More precisely, it was shown that mannosyl caps are the ligand-binding sites. Indeed, neither AraLAM- nor LM-(molecule devoid of mannosyl caps) coated microspheres significantly bind to the monocyte-derived macrophages. Moreover, ManLAM treated with an exomannosidase that hydrolyzes selectively the terminal mannosyl units, becomes unable to adhere to macrophages. Independently, using another analytical approach consisting in biotinylating ManLAM, we have shown that ManLAM from M. bovis BCG interacts with murine phagocytes. This binding is affected by the temperature, yeast mannan, and is serum-and divalent cation dependent, suggesting the involvement of the mannose receptor (112). Recently, differential binding activity of ManLAMs from virulent and attenuated strains of M. tuberculosis was observed (113). Despite the presence of mannosyl caps in all the LAMs investigated in this study, LAM from Erdman strain appears a better ligand than ManLAM from the H37Ra strain. ManLAM from H37Rv strain is intermediate. Schlesinger *et al*. (113) have suggested that the origin of these observed differences may be multiple: mannosyl capping percentage, three-dimensional structure or involvement of another receptor. The adherence of ManLAM to the mannose receptor has some repercussion on cell mediated immunity since it has been reported that a possible outcome is ManLAM presentation to double negative T cells (CD4-, CD8-) by CD1b molecules (114).

Another soluble receptor, the mannose binding protein, which looks as a key factor in innate immunity, was found to interact with lipoglycans from M. tuberculosis. Among the putative lipoglycan ligands like ManLAM, LM, PIMs from M. tuberculosis and AraLAM from fast growing Mycobacterium species, ManLAM show the highest affinity for the mannose binding protein (115). Conversely, Hoppe *et al*. (116) have shown that PIMs are the major ligand of the mannose binding protein expressed in nonphagocytic cells (Chinese Hamster Ovary fibroblasts and primary porcine aortic endothelial cells).

At last, LAM is able to insert into the membranes without the involvement of any receptor

(117). As the integration is abrogated when ManLAM is deacylated, it is presumably mediated by the phosphatidyl anchor of LAM. However this process is not inhibited by phosphatidylinositol suggesting a contribution of the polysaccharidic core.

Literature data show that, among immune cells, LAM preferentially interacts, *in vitro*, with phagocytes. This interaction leads to a regulation of cytokine release. The process is dependent on both the LAM structure (AraLAM/ManLAM; parietal LAM/cellular LAM) and the phagocyte origin and activation state.

8. PERSPECTIVE

This review highlights the structural complexity of the mycobacterial LAM and also its large spectrum of immunological functions. Paradoxically, LAM properties contribute to the immunopathogenesis but also to the protective immunity. Indeed ManLAM, unlike PI-GAM, in promoting mycobacterial uptake by mononuclear phagocytes, modulates negatively the production of oxygenated and nitric oxyde metabolites as well as inflammatory cytokines. Consequently, ManLAM, which is only present in the slow growing strains, minimizes the macrophage microbicidal activity and so contributes to the intramacrophagic mycobacterial survival. Likewise, the inability of ManLAM to induce the chemotaxis of monocytic cells prevents the recruitment of alveolar macrophages at the infection site, contributing to the pathogenesis. In contrast, ManLAM, by stimulating double negative T-cells contributes to the protective immunity against tuberculosis (for review see (13, 118, 119)). Indeed, these T lymphocytes are involved in the host defense by killing infected cells.

The precise definition of the molecular basis and the molecular mechanisms of the LAM activities remains an important challenge in the study of the tuberculosis immunopathogenesis. Investigations must be developed with the following aims: i) determination of a more precise structural model of LAMs, ii) characterization of their molecular targets on the host phagocyte cells and finally, iii) elucidation of LAM biosynthesis by the characterization of glycosyl and acyl transferases.

9. ACKNOWLEDGMENTS

We gratefully acknowledge Dr O. Humbert for comments concerning the English writing.

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Key words: Glycobiology, Receptors, Mycobacteria, Lipoarabinomannans, Cytokines

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