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PROCESSING OF MYCOBACTERIAL LIPIDS AND EFFECTS ON HOST RESPONSIVENESS

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1. ABSTRACT

One of the most important opportunistic pathogens associated with AIDS is the *Mycobacterium avium* complex. *M. avium* infections are found in up to 70% of individuals in advanced stages of AIDS. The deficiency in our knowledge of these mycobacteria presents an obstacle to the development of a rational approach for controlling these life-threatening infections in immunocompromised persons. It is apparent that *M. avium* can replicate in host macrophages and persist for long periods. During this time, various components, particularly lipids, accumulate in host macrophages and contribute to the ability of this organism to upset the cytokine homeostasis necessary for controlling infections of this type. *M. avium* lipids are immunosuppressive and can induce a variety of cytokines and eicosanoids that affect general host responses. The intention of this review is to examine the postphagocytic processing of various *M. avium* lipids with respect to their ability to alter host responses, particularly in immunocompromised patients such as those infected with HIV.

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2. INTRODUCTION

Mycobacterial infections have afflicted humanity since early recorded time. Perhaps, the best known mycobacterial infections are tuberculosis and leprosy. In recent years, another group of mycobacteria has become important in the development of human disease. Soon after medical science became aware of the human immunodeficiency virus (HIV), it was realized that an opportunistic group of mycobacteria, the *Mycobacterium avium* complex, played a major role in the progression and outcome of that viral disease. Since the early 1980's the *M. avium* complex has made an important contribution to the progression of AIDS. Historically *M. avium* has not played a major role as a human pathogen. As discussed previously (1), *M. avium* pathogenicity probably results from several contributing factors. Normally (i.e., immunocompetent host), only subclinical infections result. However, in an immunodeficient host (e.g., HIV-infected individual) the effects of these contributing factors can be critical, resulting in loss of effective host response. Thus, under the right conditions, an opportunistic pathogen such as *M. avium* can progress to the higher level of 'pathogen', and create conditions that result in higher mortality rates. For *M. avium*, this generally occurs when the patient's CD4+ population of T-lymphocytes is reduced to a level below 100 mm^2 (2, 3).

It is interesting to note that *M. avium* is the primary mycobacterial infection observed in advanced stages of AIDS, even though there are other ubiquitous opportunistic

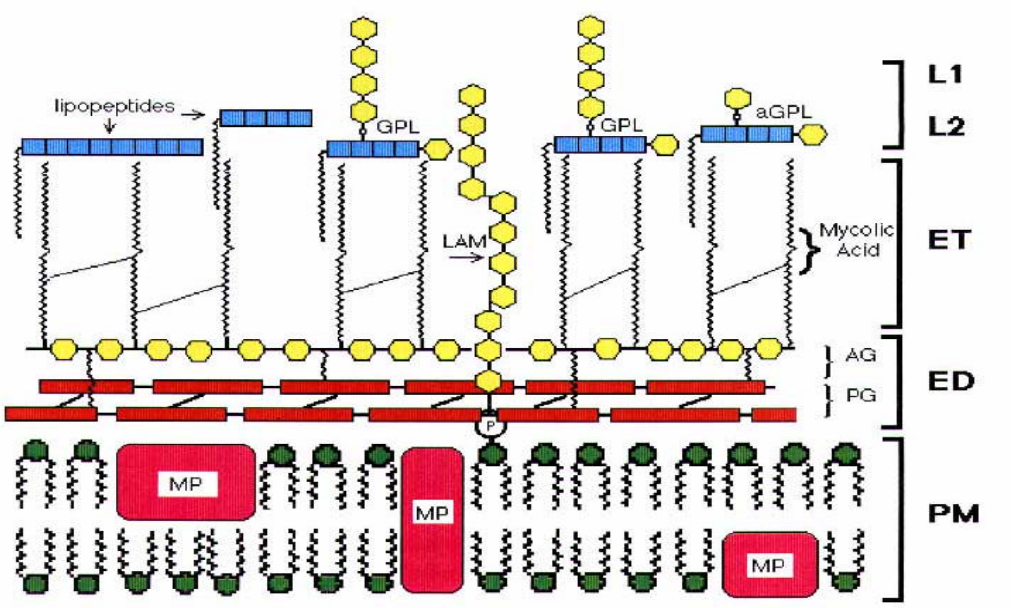

Figure 1. Model of mycobacterial cell envelope depicting the plasma membrane (PM), the electron dense (ED), electron transparent (ET), and L1 and L2 layers. This model was designed after those described by McNeil and Brennan (6), Minnikin (7, 8), and Rastogi (9), and is used here as a reference tool for discussion. For more accurate chemical and structural information, please refer to the four references given (6, 7, 8, 9). In this model, the plasma membrane is shown to contain membrane proteins (MP). Also represented are peptidoglycan (PG), arabinogalactan (AG), mycolic acid, lipoarabinomannan (LAM), lipopeptides, glycopeptidolipid (GPL), and apolar glycopeptidolipid (aGPL). This model is not drawn to scale. Further description of these structures is given in text.

mycobacterial species that can potentially co-infect AIDS patients. Reportedly, 50-70% of patients in the advanced stages of AIDS have *M. avium* infections (2, 4, 5). To understand why *M. avium* has the potential to manifest itself as a 'pathogen', and contribute to advanced stages of AIDS, it becomes important to understand the organism with regard to the various lipids associated with its growth and persistence in a host.

The purpose of this review, therefore, will be to examine the processing of mycobacterial lipids with an attempt to better understand how these events affect the immune responsiveness of the infected host. Because numerous articles have addressed the pathogenic aspects of other mycobacteria, such as *M. tuberculosis* and *M. leprae*, the primary focus of this review will be *M. avium* and its contribution to advanced stages of AIDS.

3. DISCUSSION

3.1. Mycobacterial lipids

There are a variety of mycobacterial lipids that have been described over the years. All of these will not be discussed here. The primary aim of this section is to describe the important lipids associated with *M. avium* that have the potential to affect initial and long-term responses of the host. To best understand how *M. avium* lipids can affect host responses, it is necessary to understand the basic construction of the organism's cell envelope. Several models have been proposed for the mycobacterial cell wall. For a review of these, the reader is referred to the following references (6, 7, 8, 9). Although several models have been proposed, the actual arrangement of the various cell wall components is still not completely understood, and in some cases is contradictory (10). Even so, it is possible to arrive at a usable explanation for purposes of this review.

3.1.1 Mycobacterial cell envelope

Essentially, four major layers can be envisioned for the cell envelope of mycobacteria. The first layer contains the cytoplasmic, or plasma membrane (PM, Figure 1). This layer is similar to those found in other bacteria, consisting of a permeable lipid bilayer with interacting proteins enclosing the cell's cytoplasm (6, 7, 8, 9). The second layer is sometimes called the electron dense (ED, Figure 1) layer because of its staining properties when observed by transmission electron microscopy. In this area is found the peptidoglycan/ arabinogalactan moieties that make up the basic structural component of the cell wall. Adjacent to this is a layer that appears to be electron transparent (ET layer) upon observation by transmission electron microscopy. One of the primary components in the ET layer is mycolic acid (Figure 1). There are other complex lipids located in this region, but they will not be discussed here. In the outer layers of the cell envelope a variety of components can be found, depending upon which mycobacterial species is being considered. These areas are sometimes referred to as the L1 and L2 layers, and generally appear fibrillar in nature when observed by freeze fracture or negative staining (11, 12, 13, 14, 15, 16). The outer layers are the most important with regard to initial host interaction. This is because these layers contain mycobacterial components that can induce various host responses (e.g., cytokines and eicosanoids), and help protect the mycobacteria from the detrimental effects of the phagolysosomal environment in host macrophages.

In the case of *M. avium*, the predominant superficial components found in the L1 layer are the serovar-specific glycopeptidolipid antigens (GPL) (Figure 1) (12, 14, 17, 18, 19). Also present are the structurally related apolar GPL (see aGPL in Fig. 1) (12), which lack the oligosaccharide extension from the peptide moiety (20). Other related lipids are probably located in this region because of their potential participation in GPL biosynthesis. These are recently identified lipopeptides that lack any carbohydrate moieties, but with acyl and peptide moieties similar to that of the GPL (see lipopeptide in Figure 1) (21, 22, 23).

The GPL are particularly important because they are not covalently linked to other cell wall constituents and have the ability to accumulate on the surface of *M. avium* during growth (12, 14). If that growth occurs within a host macrophage, then the GPL accumulate within the phagosomal/phagolysosomal compartments as an electron transparent zone surrounding the mycobacteria (15, 16, 17, 18, 19). As will be discussed in more detail in section *3.1.3*, the GPL are a major component in 'extractable lipid' fractions obtained from *M. avium* (24). Also found in this outermost layer is the arabinomannan extension of lipoarabinomannan (LAM), but models describing the positioning of this component are apparently contradictory (Figure 1). The internal phosphatidylinositol portion of LAM is either anchored in the plasma membrane (6), as shown in Figure 1, or positioned in the outer layer (9). Because LAM requires a more aggressive extraction procedure (25), and is not considered an 'extractable lipid' (24), it is reasonable to assume that it is more likely bound in some manner to other cell wall constituents.

As an added note, it should be realized that recent studies have revealed that the 'exocellular' layer (i.e., outer layer) of *M. tuberculosis* contains substantial amounts of polysaccharides (26). The highly branched polysaccharides consist of a poly-D-glucan and an arabinomannan, with the glucan representing up to 90% of the polysaccharides (26). These reports are important when one considers that arabinomannan, obtained from culture filtrates of *M. tuberculosis*, is immunosuppressive (27). Similar findings have not been reported for *M. avium*.

3.1.2. Lipids having biological activities

Immunosuppression has been observed with total extractable lipid derived from *M. avium* (28). Total lipid fractions can interfere with the capacity of human peripheral blood mononuclear cells (PBMC) to proliferate in response to concanavalin A (ConA), purified protein derivative of tuberculin (PPD), and phytohemagglutinin (PHA) stimulation (28). These authors suggest that the *M. avium* lipids are able to block the expression of accessory molecules on the surface of the monocytes (28).

Regarding *M. avium*, only a few lipid components have been associated with immunomodulatory properties, lipoarabinomannan (LAM), the GPL components, and a group of non-characterized 'glycolipids'. As discussed previously, LAM and GPL are located in the outermost layer of the cell envelope; however, there are apparently no papers describing the isolation and characterization of LAM from *M. avium*. Therefore, it will be assumed that the location of LAM in *M. avium* is similar to that of other mycobacteria such as *M. tuberculosis*. Location of the 'glycolipids', described by Hines, *et al*. (29), was not defined. Most other studies that have investigated immunomodulatory events have used viable *M. avium*. The immunomodulatory properties of LAM have been described in numerous papers (discussed below), most of which have utilized LAM obtained from *M. tuberculosis*. Biological properties associated with the 'glycolipids' were described in one paper, in which a 'glycolipid' fraction isolated from *M. avium* serovar 2 (i.e., *M. paratuberculosis* 18) demonstrated the ability to inhibit the killing of *Candida albicans* by activated bovine peripheral blood derived macrophages (29). The information regarding immunomodulatory properties of GPL has originated from our laboratory and will also be described below.

LAM from *M. tuberculosis* and *M. leprae* has been shown to possess a variety of biological activities including inhibition of gamma-interferon-mediated activation of macrophages (30, 31), suppression of T-cell proliferation (32, 33, 34), inhibition of mRNA synthesis encoding IL-2, IL-5 and GM-CSF in the human Jurkat T-cell line (35), and enhancement of TNF-alpha production by mononuclear cells (36, 37). In another report, investigators reportedly used LAM from both *M. tuberculosis* and *M. avium* and demonstrated stimulation of TNF-alpha in thioglycollate-elicited macrophages (38). However, the LAM was provided by another laboratory and the authors did not describe any details regarding the structure of the *M. avium* LAM (38). Two other reports describe the ability of *M. tuberculosis* LAM to interact with human and murine monocytes/macrophages (39, 40). A recent report revealed that phagocytosis of *M. tuberculosis* is dependent upon the terminal mannosyl units of LAM (41).

In some of our earlier studies with GPL, we were able to demonstrate that the surface-associated GPL are essentially nontoxic substances that stimulate a nonspecific inflammatory response when injected intraperitoneally into mice (42). However, three weeks following injection of GPL (100 µg weekly; 300 µg total), the mouse splenic mononuclear cells show a significant decrease in lymphoproliferative response to ConA, PHA, and lipopolysaccharide (LPS) (42, 43). In addition, when the GPL-treated murine spleen cells are analyzed by flow cytometry, a significant decrease in the number of cells having the Thy-1 marker is observed (43). This reduction in T-cells is due to a selective decrease in the Ly-2+ cell population (equivalent to the helper, or CD4+ subset, in humans) (43). At the time, it was proposed that the immunosuppression was due to the prolonged exposure to GPL and assumed that GPL metabolites might be responsible (43). However, since then it has not been possible to demonstrate measurable degradation of GPL in macrophage cultures using internally radiolabelled GPL. Our studies have utilized both murine macrophages (44, 45), and murine and human monocytic cell lines (not published). This implies that the GPL are relatively inert and affect host responses as intact substances.

A group of 'polar glycopeptidolipids' from *M. chelonae* have been shown to possess biological activity. Although not structurally characterized, these 'polar glycopeptidolipids' were extracted in a manner similar to that

Table 1. Comparison of various *M. avium* extractable lipid fractions with regard to carbohydrate content and ability to induce various cytokine and eicosanoid responses in macrophages.

Lipid Fraction	% Carbohydrate (mean ± SEM) [a]	% 6-deoxyhexose (mean ± SEM) [b]	TNF-alpha [d,e]	IL-1 [e]	Response [c] IL-6 [e]	PGE_2 [d,e]	TXB_2 [e]
Total	13 ± 0.8	10 ± 0.5	+	+	+	+	+
100% Chloroform	< 5	NT	-	-	-	-	-
3 %	< 5	NT	-	-	-	-	-
5-7 %	46 ± 3.5	41 ± 3.5	+	-	-	+	-
7-8% GPL	35 ± 1.8	22 ± 1.2	+	+	+	+	+

Total carbohydrate ([a]) and 6-deoxyhexose ([b]) contents were determined by procedures described by (111) and (112), respectively. Some experiments involved human peripheral blood mononuclear cells ([d]) (54, 55, 56, 63), whereas others involved the human Mono Mac 6 monocytic cell line ([e]) (58).

used for *M. avium* GPL (46). In a series of publications, Pilet and associates, reported that 'polar glycopeptidolipids' from *M. chelonae* (1) have adjuvant activity, with regards to protective effects of an inactivated influenza vaccine (46), (2) can be used as a hematopoietic growth factor (47), and (3) can increase the resistance of mice against lethal infection with *Candida albicans*, apparently mediated by the glycopeptidolipids' ability to induce hyperleukocytosis (48). Thus, it appears that GPL components have a variety of biological activities.

As for other mycobacterial lipids found in the outer cell envelope, there is no direct evidence to indicate whether they are degradable or not. However, electron microscopic examination of *M. leprae* infected tissue indicates that the antigenic phenolic glycolipids (49) are deposited on the surface of the organism and accumulate in macrophages much the same as the GPL of *M. avium* (15). From this, one might assume that these glycolipids are also resistant to degradation. This is important from a host-parasite viewpoint because *M. leprae* phenolic glycolipids are immunosuppressive (50), can reduce monocytic oxidative responses (51), and can inhibit proliferation of human blood mononuclear cells in a nonspecific manner (52). This might partially explain why *M. leprae* loaded macrophages are refractory to activation by gamma interferon (53). Phenolic glycolipids from *M. bovis* BCG and *M. kansasii* are similar in their ability to inhibit proliferation of human mononuclear cells (52).

In an attempt to better understand specifically how mycobacterial lipids affect host responses, we have conducted a series of experiments using *M. avium* lipids. Initial studies using human peripheral blood mononuclear cells (PBMC) have revealed that total extractable lipid fractions from *M. avium* can induce PGE_2 and TNF-alpha (54, 55). When various lipid fractions are obtained by column chromatography and tested with PBMC, the major fraction showing immunomodulatory properties is the GPL fraction (54, 55, 56). Other studies in our laboratory have shown that the GPL fraction causes the release of soluble factors from murine peritoneal macrophages that produce suppression in lymphoproliferative responsiveness of splenic mononuclear cells (56). It is interesting to note that a recent publication reported a similar finding with live *M. avium* infected murine macrophages; i.e., production of a soluble inhibitory factor (57). In their study, the investigators gave supportive evidence to suggest that the inhibition was due to IL-6 (57).

A recent continuation of our studies, using the Mono Mac 6 human monocytic cell line, has revealed that *M. avium* total extractable lipid and GPL can induce all three of the inflammatory cytokines (i.e., TNF-alpha, IL-1, and IL-6), and in addition, PGE_2 and TXB_2 (58). Furthermore, treatment of the human Jurkat T-cell line with total extractable lipids and GPL fractions from *M. avium* results in inhibition of IL-2 production (59)(manuscript in preparation). Thus, the GPL have a variety of biological activity that could influence host responses. An explanation of the lipid fractions and a summary of biological activities associated with each fraction are given in Figure 2 and Table 1, respectively.

3.1.3. Separation of extractable *M. avium* lipids by HPLC

Figure 2 represents an HPLC separation of the various extractable lipids that can be obtained from *M. avium*. It should be emphasized that extractable lipids, in this case, are those that are not covalently linked in the cell envelope (24); i.e., those components associated with the outer L1 and L2 layers and most likely to accumulate as *M. avium* grows (see 3.1.1). To facilitate the identification of each fraction, the lipids were internally radiolabeled with [^{14}C]-phenylalanine because phenylalanine is in the peptide moiety of all GPL components of *M. avium* and is directly linked to the fatty acyl moiety (60). HPLC fractions were then monitored by means of a solid system radioisotope detector (Beckman). Further identification of fractions was conducted by using thin-layer chromatographic techniques previously described. For references to describe these procedures, the reader is referred to the following manuscripts (23, 44, 54, 61).

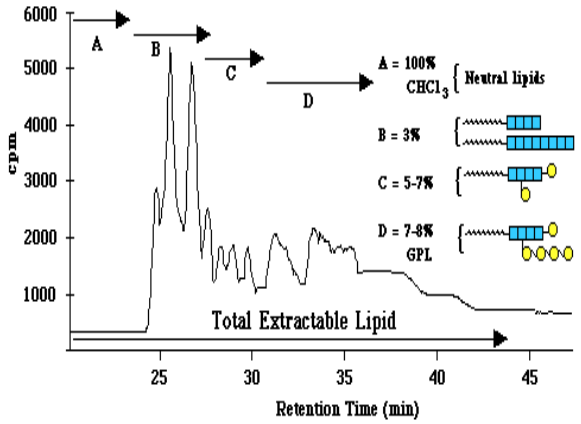

Figure 2. Representation of HPLC separation of *M. avium* extractable lipids. Lipids were separated on a Beckman Ultrasphere SI analytical column, as described previously (54). Lipid samples were separated in a mobile phase of 100% chloroform for 10 min followed by separation in a 40-min gradient of 0 to 10% methanol in chloroform, at a flow rate of 1.0 ml/min. After the gradient, l0% methanol in chloroform was maintained for 10 min and then returned to 100% chloroform over 10 min (54). Areas which coincide with eluted fractions are given as 100% chloroform (A), 3% methanol in chloroform (B), 5 to 7% methanol in chloroform (C), and 7-8% methanol in chloroform, containing glycopeptidolipid (GPL) (D), such as those previously described (12, 113). Modified from reference 54 with permission.

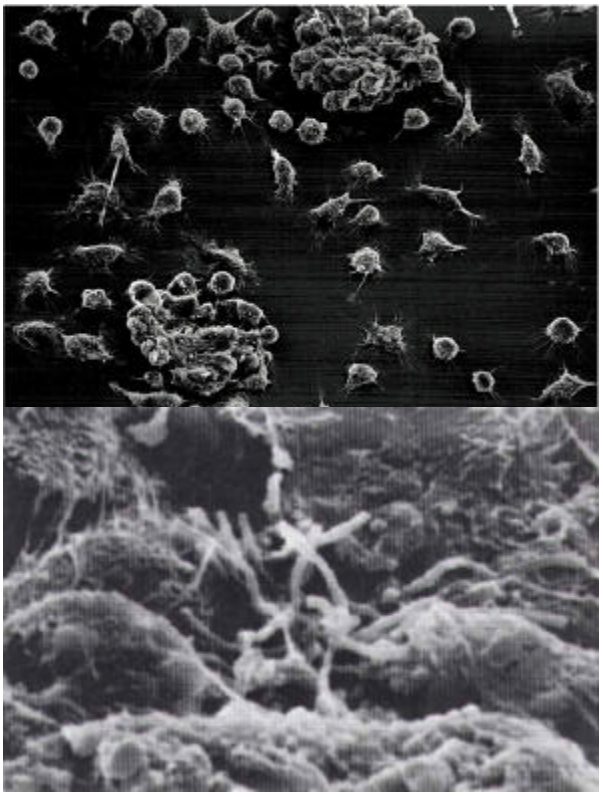

Figure 3A (top) and B (bottom). Scanning electron micrographs of mouse peritoneal macrophages infected with *M. avium* serovar 4. Figure 3A was taken at 400x and Figure 3B at 4000x.

3.1.4. Biological activities of HPLC fractions

Table 1 lists the lipid fractions obtained by HPLC separation with regard to their ability to induce various immunomodulators along with respective carbohydrate and 6-deoxy-hexose content (information taken from (54, 56, 58)). Quantitation of 6-deoxy-hexose was performed because the sugars associated with GPL are that type of carbohydrate (12, 20, 54, 62). From this information it is apparent that there is a direct correlation with carbohydrate content and the ability of the lipid fractions to induce secretion of immunomodulators. This correlation is further supported when one considers the recent findings reported by Vergne, *et al.* (63), who have shown a direct relationship between carbohydrate content of GPL components and ability to interact with membranes (see discussion below, 3.4).

3.2. Host response to *M. avium*

3.2.1. Initial interaction

Mycobacteria are facultative intracellular parasites. Therefore, following infection either through the gastrointestinal or respiratory tract, they are taken up by macrophages where they reside during the infectious disease process (4). Mycobacteria can also be trafficked to other sites throughout the reticuloendothelial system where they generally persist and multiply (4). As an example of the interaction between host macrophages and *M. avium*, an *in vitro* infection with mouse peritoneal macrophages will be used. Figures 3A&B are scanning electron micrographs depicting a typical initial interaction between host macrophages and *M. avium*. In this case, mouse peritoneal macrophages have been infected with *M. avium* serovar 4. The macrophages begin to migrate into clusters and phagocytose the *M. avium*, even without opsonizing antibodies (Figures 3A&B). Following phagocytosis, the infected macrophages can be stained by means of the Ziehl-Neelsen acid-fast staining procedure, and *M. avium* observed as acid-fast (red) bacilli located within the confines of the macrophage (Figure 4A). After a prolonged infection (seven days in this case), the cells can be stained again and one can appreciate the ability of the mycobacteria to survive and grow within the host macrophage (Figure 4B). In the short period of one week, the mycobacteria have grown from an initial infection ratio of 1-10 per macrophage (Figure 4A) to >100 per macrophage (Figure 4B). Although this is an *in vitro* representation of the infection, similar observations can be made in tissue samples of AIDS patients co-infected with *M. avium*. In some cases, tissue loads can reach as high as 10^9-10^{10} mycobacteria per gram of tissue (64).

3.2.2. Later stages

Control of *M. avium* by the host depends upon a functional cell mediated immune response programmed by proper communication between two important CD4+ lymphocyte populations that have been called Th1 and Th2 (65). An important host mechanism that allows CD4+ T cells to mediate antimycobacterial activity is the production of cytokines (66). Cytokines characteristic for Th1-type responses include primarily IL-2 and IFN-gamma, whereas those characteristic for Th2-type responses include IL-4, IL-5 and IL-10 (67). It has been reported that immunity to *M. avium* is dependent upon the induction of protective CD4+ T cells

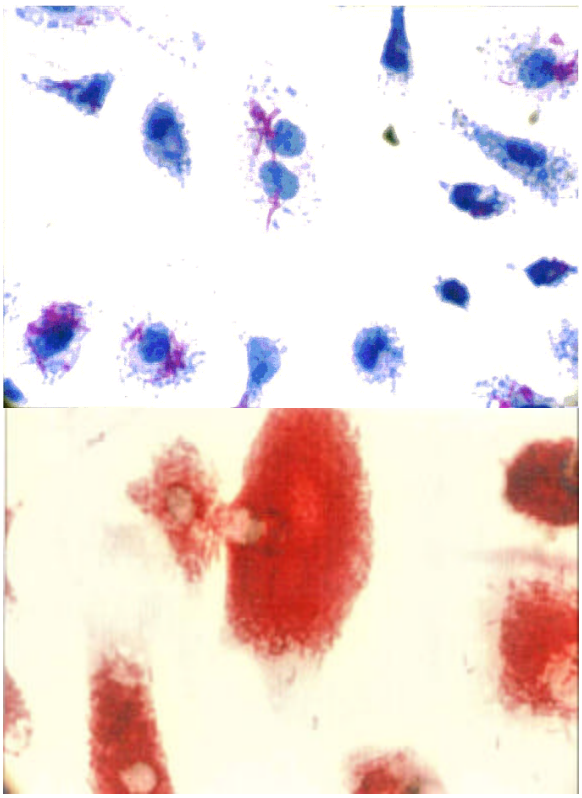

Figure 4A (top) and 4B (bottom). Light microscopic photograph of mouse peritoneal macrophages infected with *M. avium* serovar 4 at Day 0 (Fig. 4A) and Day 7 (Fig. 4B). Infected macrophages were acid-fast stained and examined by light microscopy at 630x.

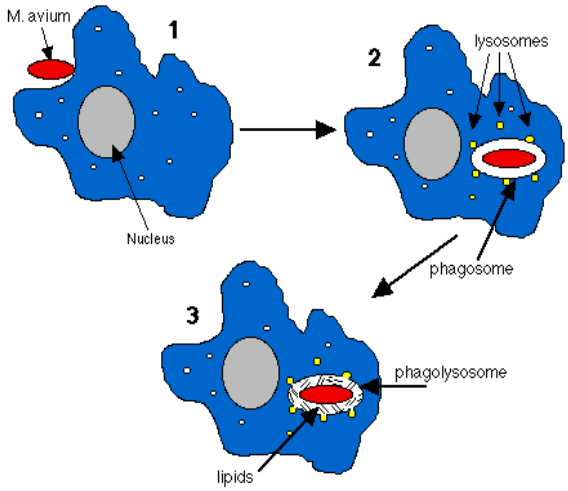

Figure 5. Engulfment of *M. avium* by host macrophage. Step 1 represents initial attachment of *M. avium* to host macrophage. Step 2 represents *M. avium* within phagosomal compartment of macrophage and lysosomes. Step 3 represents *M. avium* within phagolysosome, containing lipids that accumulate throughout the disease process. Figure is not drawn to scale.

(primarily with a Th1 type response). This leads to protective immunity that is dependent upon IFN-gamma and TNF-alpha (65, 68, 69). More importantly, the early phase of the host response is CD4+ T-cell-independent, and only shows a CD4+ T-cell-dependent phase of immunity when high bacterial loads are achieved (70). Thus, by using CD4+ T-cell-deficient mice (70), or CD4+ T-cell-depleted mice (70, 71), it was demonstrated that the dependency for CD4+ T-cells does not become apparent until 2-3 months following infection, when *M. avium* loads approach 10^8 and 10^{10}. In other words, in mice depleted of CD4+ T-cells, a *M. avium* infection apparently does not compromise a host until late in the disease when high levels of the organism become manifest. This is probably analogous to what occurs in HIV-infected individuals as the disease progresses through the various stages of CD4+. Although the specific reasons for this dysfunction have not yet been defined, one attractive possibility is that accumulating *M. avium* lipids play an important role in exacerbation of disease by their ability to induce immunomodulatory components. This could result in an imbalance of the cytokine network and therefore contribute to the overall outcome of the disease process.

3.2.3. Cytokines important in host response

Cytokines are a family of small (6-10 kDa) glycosylated polypeptides that include interleukins (ILs), interferons (IFNs), tumor growth factors (TGFs), tumor necrosis factors (TNFs), and colony stimulating factors (CSFs) (72, 73, 74). This highly interactive family of pleiotropic cell-regulatory molecules operates within the context of a network in which upregulation and downregulation activities continually contribute to the interaction between different cell types (74). Examples of this regulation would be TGF-beta, which depresses the synthesis of IL-1, IL-2, IL-6, IL-7, TNF-alpha and other proinflammatory cytokines, and IL-10 that downregulates the production of TNF-alpha, IL-1, IL-6, IL-8, GM-CSF and G-CSF, but upregulates the synthesis of IL-1 antagonists (75). Cytokines such as IL-1 and TNF-alpha stimulate the production of each other, as well as IL-2, IL-6, and IL-8; they are also strongly synergistic for the induction of GM-CSF production (75). The cytokine network constantly attempts to restore homeostasis in infected tissue and dysfunction in these regulatory networks results in an imbalance that can lead to exacerbation of diseases (75). Cytokines are extremely potent; nanomolar or picomolar concentrations are all that are necessary for activity (75).

Although a comprehensive understanding of *M. avium* 's pathogenesis has not yet evolved, it can be concluded that the initial interaction of the immune system with *M. avium* is multifactorial (1, 76, 77). Cytokine responses that have been evaluated include IL-1 alpha, IL-1 beta, IL-6, IL-10, TNF-alpha, and TGF-beta (57, 78, 79, 80, 81, 82, 83). Other studies report an immunosuppressive capacity of *M. avium* associated with prostaglandin E_2 (PGE_2) (84, 85, 86, 87, 88).

3.3. Postphagocytic events and processing of mycobacterial lipids

3.3.1. Summary and model of phagocytic events

After initial infection, *M. avium* is phagocytosed by host macrophages. Initial steps include the attachment of *M. avium* to the macrophage (step 1, Figure 5). Following phagocytosis, the mycobacteria are enclosed within a phagosomal compartment (step 2, Fig 5). The lysosomes then begin to fuse to the phagosome to form phagolysosomal

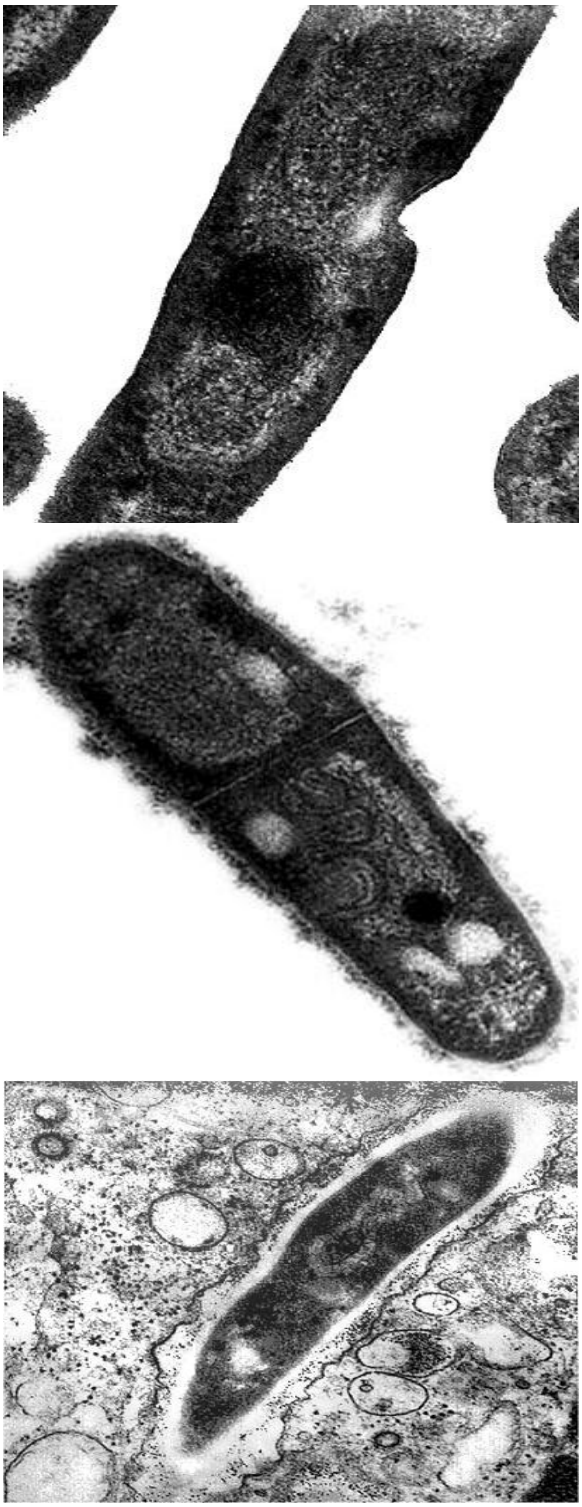

Figure 6. Electron micrograph of *M. avium* serovar 4, non-labeled (A; top) and double-labeled, using rabbit anti-GPL and goat anti-rabbit IgG ferritin conjugate (B; middle&C;bottom), as previously described (19). Figure 6C represents post-phagocytic localization of ferritin-labeled GPL surrounding *M. avium* within phagosomal compartment. Modified from reference 19 with permission.

compartments (step 3, Figure 5), which under normal conditions is enough to destroy an invading organism. However, in the case of *M. avium* degradation does not occur because either fusion of the lysosomes is prevented (89), or the organism resists lysosomal degradation due to the presence of a protective superficial sheath (17). Or, perhaps both of these mechanisms apply. As the infection progresses, *M. avium* continually deposits various lipids on its surface throughout the disease process (step 3, Figure 5). As discussed below (see *3.3.2* and *3.3.3*), most of the evidence so far indicates that GPL components are the predominant lipid deposited in the phagosomal compartment.

3.3.2. Development of electron-transparent zone

Early studies by Draper and associates initially described the appearance of the so called 'electron-transparent' zone that developed as *M. avium* grew within phagosomal compartments of host macrophages (14, 17, 18). It has been shown that *M. avium* superficial components (e.g., GPL and related lipids) (19) accumulate in macrophages (14, 15). Postphagocytic localization of GPL can be observed by transmission electron microscopy, using ferritin-labeled antibody directed to the GPL (Figures 6A-C) (19). In Figure 6B the superficial GPL have been pre-labeled with rabbit polyclonal antibodies specific for the GPL. The second label is ferritin-conjugated goat anti-rabbit antibody that reveals the superficial localization of the GPL following phagocytosis by mouse peritoneal macrophages (Figure 6C). A subsequent study by Rulong, *et al.* (16) used electron microscopy to demonstrate substantial accumulation of GPL material in liver macrophages following long term infection in mice. Thus, the ability of GPL to accumulate in host macrophages has been substantiated by several investigators.

3.3.3. Persistence of lipids in macrophages

It is not surprising that GPL have the ability to persist in host macrophages because these superficial lipids are relatively inert to macrophage degradation (44, 45). Thus, it is very reasonable to assume that GPL components would accumulate in chronic stages of *M. avium* infections. Although no one has yet determined what specifically happens to these lipids throughout an infectious disease process in humans, it is very likely that because of inertness, they begin to accumulate within various sites of the reticuloendothelial system. Indeed, some of the most common sites of *M. avium* involvement in humans are the lymph nodes (74%), spleen (74%), and liver (52%) (90). This issue becomes important when one considers that disseminated *M. avium* infections in AIDS patients can generate serum levels as high as 10^4 CFU/ml (91) and loads of 10^9-10^{10} mycobacteria per gram of tissue (64).

As our studies have revealed, 10^8 to 10^{10} *M. avium* can potentially produce approximately 0.18-18 mg of GPL *in vitro* (54). This does not take into account the accumulating amounts of GPL and related lipids that would occur in an infected host. Even so, this is a sufficient quantity of GPL to induce a variety of immunomodulatory components such as TNF-alpha and PGE_2 (54, 55), as well as IL-1 beta, IL-6, and TXB_2 (58). It is interesting to note that in HIV-infected patients who are co-infected with *M. avium*, blood cells release higher levels of TNF-alpha, IL-1, and IL-6 than blood cells from HIV-infected patients not co-infected with *M. avium* (92,

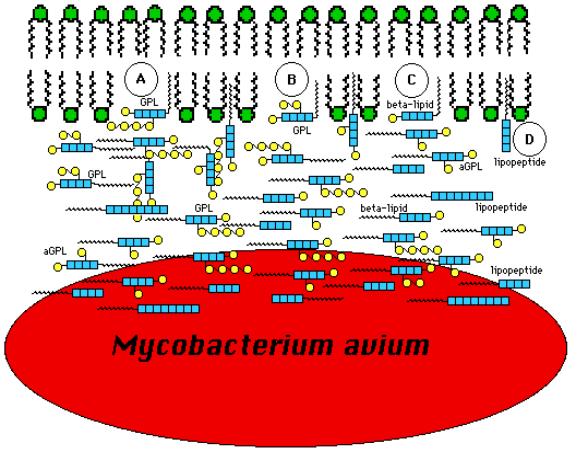

Figure 7. Representation of various superficial lipids that would likely accumulate as a result of *M. avium* growth in the phagolysosomal compartment of host macrophages. The plasma membrane of a phagosomal/phagolysosomal compartment is represented at the top of the figure.Various lipids represented in figure include the serovar-specific GPL components (GPL), apolar GPL, the lipopeptide fragment produced by beta-elimination of GPL (beta lipid) and lipopeptides of the kind recently described (1, 21, 22, 23). A *M. avium* cell is represented at the bottom of the figure. This figure is not drawn to scale.

93). That anomaly does not occur in noninfected individuals or those in the early stages of HIV-infection (92).

3.4. Model for lipid interactions with host cells

As proposed by Lanéelle and Daffé (76), there is no 'coherent model to explain mycobacteria pathogenicity'. However, as these authors suggest, interactions between mycobacterial lipids and host membranes might be an interesting hypothesis to help explain mycobacterial pathogenesis (76). This hypothesis is derived from the fact that mycobacteria produce substantial quantities of lipids (76) that have the ability to penetrate membranes (94). Other evidence for the ability of mycobacterial lipids to alter membranes has been reported by several investigators, including the following: (1) Stewart-Tull, *et al.* (95), showed that a peptidoglycolipid from *M. tuberculosis* can increase permeability of liposomes. (2) Roozemond and associates (96, 97), demonstrated the ability of total lipid extract from *M. bovis* to decrease the 'fluidity' of the membranes of natural killer cells. (3) Sut, *et al.* (98), showed that two trehalose derivatives can cause rigidification of the fluid state of liposomes and can inhibit mitochondrial oxidative phosphorylation and, that peptidoglycolipids (i.e., similar in structure to the GPL discussed in this review) have the ability to cause increased leakiness in liposomes and the ability to inhibit oxidative phosphorylation "...in a manner resembling that of classical uncouplers...". (4) Vergne, *et al.* (63), demonstrated the ability of mycobacterial GPLs to become inserted into phospholipid monolayers. From these representative studies, it is possible to formulate a plausible explanation for the ability of *M. avium* lipids to alter host responsiveness by interaction with cell membranes.

Figure 7 is a representation of the postphagocytic accumulation of the various lipids that would be expected as *M. avium* grows within host macrophages. In this representation, *M. avium* is situated within the phagosomal/phagolysosomal compartment of the host macrophage. The membrane of the phagosomal/phagolysosomal compartment is positioned in the upper portion of Figure 7 and the *M. avium* lipids are positioned between the mycobacterial cell and the membrane. As the lipids accumulate, they form the extracellular sheath (L1 layer) that is observed as an electron-transparent zone upon examination by transmission electron microscopy (see *3.3.2*). The lipids then begin to interact with the membrane, depending upon their ability to insert within the phospholipid bilayer. It is possible that this accumulation of various lipids might also play an important role in the resistance that this organism shows to various antimycobacterial drugs.

It has been shown by Lanéelle and coworkers that mycobacterial lipids such as GPL interact with membranes, depending upon the carbohydrate residues (63), and increase permeability and inhibit oxidative phosphorylation (76, 98). Glycopeptidolipid components containing 3 or 1 carbohydrate moieties, such as those represented in (B) and (C), respectively (Figure 7), are better able to interact with the membrane than those containing no carbohydrate moieties (D) (Figure 7), and are judged to be active by compression isotherms (63). We can assume from those studies that components such as the serovar-specific GPL, represented as (A) (Figure 7), will also interact with membrane systems. However, lipopeptides such as those represented by (D) (Figure 7), can apparently interact only slightly by insertion of the acyl moiety, but are not active as determined by compression isotherms (63). These authors have proposed that accumulation of GPL components in infected macrophages would therefore help *M. avium* to survive by interfering with membrane-linked functions in the host cell (63).

It should be noted that lipids such as those represented in (C), (Figure 7)(i.e., beta-lipids) are derived from chemical modification of GPL components; the oligosaccharide is removed by alkaline-catalyzed beta-elimination (20, 99). The beta-lipid fragment obtained from *M. avium* GPL has been shown to be the immunosuppressive portion of the molecule (99) and can cause damage to host macrophages (56). However, the presence of similar molecules in a host has yet to be demonstrated. Even so, it is possible that similar types of lipids exist, either as precursors of GPL biosynthesis or as byproducts of GPL degradation. Although our studies have suggested that GPL are not readily degraded in macrophages *in vitro*, it is still possible that some degradation might take place in a host over a long period of time. If so, then it is very possible that lipids similar to the beta-lipids might exist in *M. avium* chronic infections.

Postphagocytic events in chronic stages of the disease can only be hypothesized now. Because of their ability to interact with membranes, it is very likely that the GPL and related lipids would eventually interact with immunologically important cells in the vicinity of the macrophage that had engulfed them initially. As the lipids began to accumulate, they might be eliminated

from the macrophage by normal processes or released as the result of cell death. Following elimination from host macrophages, the lipids would then most likely interact with other host cells in a manner similar to that described above and in Figure 7. Further studies will be required to define these parameters in a human infection.

3.5. Progression of AIDS and *M. avium's* contribution to the exacerbation of HIV pathogenesis.

3.5.1. HIV pathogenesis

To understand the contribution of mycobacterial products to the progression of AIDS, it is necessary to first understand, at least briefly, the progression of an HIV-infection to the advanced stages of AIDS. The features of HIV pathogenesis are discussed by Levy (100). It is thought that the virus initially enters a patient primarily by infecting either activated T cells, resident macrophages, or mucosal cells in either the bowel or the uterine cavity (100). In the early days of the infection, it is postulated that the virus replicates to high levels in macrophages in the lymph nodes(100). Within one month the viremia is usually diminished because of the immune response (100). Although viral replication persists in the body (i.e., in lymph nodes and peripheral blood mononuclear cells), viral loads in the blood are generally low(100). The CD4+ cell numbers decrease over a period of time(100). Loss of antiviral response occurs and the patient develops advanced stages of AIDS(100). The number of CD8+ cell response begins to decline much sooner than the CD8+ cells themselves (100). As discussed by Levy (100), the loss in antiviral response over time "...is one of the unresolved mysteries of this infection."(100). It is clear that macrophages and T-lymphocytes are important in the continuation of HIV replication and progression to the later stages of AIDS.

Although information is incomplete, it is apparent that cytokines play an important role in HIV pathogenesis and the progression of AIDS. A multitude of cytokines can be demonstrated in immunoresponsive cell types, but only a few appear to be consistently associated with the continued persistence of HIV and subsequent exacerbation of viral loads in AIDS patients. Those cytokines include TNF-alpha, GM-CSF, IL-1, and IL-6, generally produced by monocytes, and TNF-beta, produced predominantly by T and B cells (reviewed in (101)). It is thought by some investigators that TNF-alpha and TNF-beta are the key mediators of AIDS pathogenesis (101). Those same investigators feel that opportunistic infections result in higher levels of TNF-alpha and TNF-beta, which in turn contribute to a decline in CD4+ cells by potentiation of cytotoxic effector functions. As a result, HIV-infected CD4+ cells, as well as CD4+ cells covered with HIV antigen, will be killed by cytotoxic effector cells (101). It is possible that TNF-mediated activities might even hasten the progression to full-blown AIDS (101). Other cytokines involved in this proposed model include IL-1 and IL-6, primarily produced by monocytes/macrophage following infection with HIV (101). Evidence regarding Th1 and Th2 T-cell populations (explained below) suggests an importance for other cytokines such as IL-2 and IFN-gamma (associated with Th1 responses), as well as IL-4 (associated with Th2 responses) (100). In addition, it has been demonstrated that a factor released from activated CD8+ cells can suppress the replication of HIV; this factor is now called IL-16 (102). Lastly, IL-2, a factor released from stimulated monocytes and macrophages, may also be important because of its ability to mediate the development of Th1-specific immune responses (i.e., IFN-gamma) (103, 104, 105, 106). Apparently the cytokine network implied in the development of HIV pathogenesis is very complex.

Also important in the development of AIDS are the numbers and proportions of lymphocytes and their related cytokines. Two major groups of T-helper (CD4+) lymphocytes are apparent based upon their cytokine profile, Th1 and Th2. The Th1 cells produce IL-2, IFN-gamma, and lymphotoxin (TNF-beta), whereas Th2 cells produce IL-4, IL-5, IL-10 and IL-13 (67, 107, 108). The Th1 cytokine responses are important in the development of both cell-mediated and humoral immunity and the Th2 cytokine responses provide optimal help for humoral immune responses (108). As discussed above, the Th1 cytokine profile appears to be the one selectively induced in mycobacterial infections (109) and important in controlling *M. avium* (110).

3.5.2. Contribution of *M. avium* to progression of AIDS

If *M. avium* lipids have the ability to alter host responses via disruption of cytokine networks (particularly those associated with Th1-type responses), it is very plausible that this alteration could play a role in the eventual outcome of AIDS. Thus, initial infections with *M. avium* might only be subclinical, because it is not a frank pathogen and lipid would not have accumulated to sufficient levels to alter normal host responses. The patient might even be able to control the infection for several months or years as the CD4+ T-cell population declined. However, as the organism would continue to grow and deposit more immunomodulatory lipids, it is probable that host-parasite interactions would eventually be affected. This would be particularly important as the number of CD4+ cells began to decline because cytokine homeostasis would be compromised even further. If components such as the GPL have the ability to decrease CD4+ cells in a host, as suggested by our previous study in mice (see above, *3.1.2*) (43), the problem would be augmented. In turn, as the CD4+ population declined, the proper Th1-type cytokine responses would be modified and the ability of the host to mount an effective immune response compromised.

4. PERSPECTIVE AND SUMMARY

There is much work left to do to completely understand how *M. avium* contributes to the general outcome of the host response to HIV infections. However, *M. avium* clearly has the ability to affect various phases of host responsiveness. This is particularly important in advanced stages of the infection when mycobacterial loads are elevated and host lymphocyte populations have lost the appropriate ratios necessary to equilibrate effective cell mediated immune responses. Although there are probably other mycobacterial components associated with the growth of *M. avium*, it is logical to assume that lipids would be the most likely element to affect host responsiveness over a long period. This would primarily be due to the lipophilic nature and low-level of biodegradability generally associated with the complex lipids produced by mycobacteria. Because of

their innate properties, certain *M. avium* lipids can interact with host membranes and generally disrupt overall function and stability of the cell associated with that membrane system. Disruptions like this would include not only the initially infected macrophage, but would also eventually include surrounding bystander cells such as T and B lymphocytes.

In addition, the ability to induce various cytokines and eicosanoid components allows *M. avium* lipids to affect the general homeostasis of the complex cytokine network necessary to program effective host responses to intracellular pathogens such as mycobacteria. Although the emphasis of this review has been the GPL components of *M. avium*, it is important to realize that other *M. avium* components may also prove to be important in the long-term effect on HIV-infected individuals. Further studies are necessary to complete the total picture and to define more fully *M. avium*'s contribution to pathogenesis in AIDS.

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