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MYCOBACTERIUM AVIUM INTERACTION WITH MACROPHAGES AND INTESTINAL EPITHELIAL CELLS

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1. ABSTRACT

Mycobacterium avium is an environmental microorganism that is adapted to live both in the environment (mainly in water and soil) and in bird, fish and mammal hosts. In humans, *M. avium* infection is seen in patients with some sort of immunosupression, such as patients with chronic lung disease, and Acquired Immunodeficiency Syndrome. More recently, other populations were shown to be at risk to develop *M. avium* disease.

For the majority of time, humans acquire *M. avium* through the intestinal tract where the bacterium comes in contact with and translocates the intestinal mucosa. *M. avium* possesses a unique manner to interact with the intestinal mucosa, and, following invasion, can enter and survive within macrophages and monocytes. Although *in vitro* entry seems to be dependent on binding to the complement receptor, this finding has not been observed *in vivo* where the bacterium appears to enter macrophages by alternative mechanisms. The bacterium appears to trigger little inflammatory response, and is able to adapt itself to different environments in the host.

2. INTRODUCTION

The lifestyle of bacterial pathogens requires them to establish infection in the presence of the host immunity. Upon entrance of *M. avium* into the host, a variety of interactions are initiated and the outcome will depend on a number of factors. A pathogen damages the host and compromises the integrity of cells.

M. avium is an environmental organism encountered in water and soil and has been isolated from birds, swine, cattle, and non-human primates. Infections caused by organisms of the *M. avium* complex are mainly pulmonary in immunocompetent patients and disseminated in immunosuppressed patients. Pulmonary infections are diagnosed in patients with predisposing lung conditions such as pneumococcosis, silicosis, cured tuberculosis and chronic obstructive lung disease (1, 2).

M. avium has been identified as causing disease in patients who use alcohol (3). In addition, *M. avium* infection has been increasingly documented in middle aged women most of whom have structural changes in the chest (4). Lymphadenitis in children is also frequently caused by organisms of the *Mycobacterium avium* complex (5).

In AIDS patients, *M. avium* is associated with disseminated disease in 40-50% of the patients with fewer than 50 $CD4^+$ T cells/mm3 of blood.

3. MECHANISMS OF INFECTION

3.1. Attachment and Invasion of Mucosal Surfaces

While in AIDS patients current evidence suggests that the majority of infected individuals acquire *M. avium* through the intestinal tract, in non-AIDS individuals the most likely route of infection is the respiratory tract (1, 2). In both cases, however, *M. avium* comes in contact with the host's mucosa before establishing infection. Once in the alveolar space, the bacterium can colonize and infect both alveolar macrophages and type II alveolar epithelial cells. In fact, recent studies have shown that *M. avium* interacts with type II alveolar epithelial cells (6) and potentially can use this route to translocate across the mucosal barrier.

The ability of mycobacteria to bind to and invade epithelial cells was initially demonstrated in studies by Shepard working with HeLa cells (7). *M. avium* is also capable of entering into alveolar epithelial cells which may have an important role in the mechanisms of infection. A study, however, has failed to show that *M. avium* strains are capable of multiplying within alveolar epithelial cells (8) although evidence for slow replication exists (6) Nonetheless, more studies are necessary to establish the role (if any) of invasion of alveolar epithelial cells in the pathogenesis of mycobacterial infection of the lung. In addition the pathway used by *M. avium* to infect epithelial cells is presently unknown, although it has been shown that *M. tuberculosis* binding can be significantly inhibited by anti-vitronectin receptor and anti-β1 integrin antibodies (6).

In addition, Schorey, *et al*, have shown that uptake by bladder epithelial cells occurs through binding to fibronectin (9). The uptake of *M. tuberculosis* by epithelial cells was shown to be both microtubule and microfilament dependent (6).

Colonization of the intestinal tract by *M.avium* is a common finding in a large number of patients with AIDS. Colonization has been shown to precede bacteremia by several months (10) and specifically in this group of patients *M. avium* is seen in the lamina propria infecting submucosal macrophages (11).

We and others have shown that *M. avium* can enter the intestinal epithelial cells *in vitro* and the intestinal mucosa *in vivo* (12, 13) The bacterium interacts with the intestinal brush border by an uncharacterized manner and subsequently establishes contact with the epithelial cell membrane. Host actin is required for bacterial uptake since cytochalasins inhibit entry (12). In addition, host signal transduction mechanisms appear to be necessary for bacterial internalization, and pharmacological inhibitors of host tyrosine kinase block host cell signaling prevent bacterial uptake (12).

Recently, it was demonstrated that the ability of the bacterium to invade intestinal cells is controlled by environmental cues. Both high osmolarity and low oxygen tension in the environment significantly increase the ability of *M. avium* to invade intestinal epithelial mucosal cells (14). Incubation of the bacteria with a sub-inhibitory concentration of amikacin, to inhibit protein synthesis, abolishes the effects of environmental factors on *M. avium* internalization, indicating that protein synthesis is required (14). Therefore, the process of invasion of intestinal epithelial cells is regulated by environmental factors such as oxygen tension, osmolarity and temperature (12, 14). Invasion in vitro and in vivo is significantly greater when bacteria are incubated at 37°C instead of 30°C prior to the assay (12, 13).

The entry of pathogens into intestinal mucosal cells has been shown to be followed in the majority of the cases in which it was examined, by the release of cytokines and chemokines, such as TNF-α, GM-CSF, IL-6, MIP-1α, RANTES and IL-8. However, release of chemokines by epithelial cells does not immediately follow invasion by *M. avium*,. Its secretion appears to be only delayed from two to three days in oropharyngeal cells, but is completely suppressed in HT-29 intestinal epithelial cells (15).

The suppression of the production of chemokines such as IL-8, a CXC chemokine, by *M. avium* when entering polarized monolayers of intestinal cells, in contrast to invasion of intestinal cells by *Salmonella typhimurium* and other pathogens, suggests that this phenomenon may represent a mechanism of pathogenesis aimed to remain unnoticed until the infection has been established. The manipulation of the host immune response by blocking chemokine production may explain the lack of inflammatory response in the intestinal wall (neutrophil infiltration followed by the presence of lymphocytes in the mucosa and submucosa) during the first days after oral ingestion of the organisms (16). In fact, intestinal epithelial cells infected with *M. avium* do not release RANTES and MIP-1β, two C-C chemokines, proteins chemotatic for lymphocytes and monocytes (15).

After entry into the intestinal cells, *M. avium* bacteria are seen within cytoplasmic vacuoles and ultimately infect macrophages in the lamina propria (11, 17) although the mechanisms involved in the translocation through the epithelial cell layer remain unknown. There is no current evidence to support that *M. avium* while within the intestinal epithelial cells can be found outside vacuoles (18). The vacuoles that contain more than one organism early in the infection, ultimately segment and as result vacuoles in cells infected for longer than 72 h contain single bacterium within. In epithelial cells, *M. avium* lives in larger vacuoles than in macrophages although the meaning of this observation is unknown (18). The data available suggest that *M. avium*'s vacuole in macrophages goes through several stages of maturation since *M. avium* synthesizes a number of proteins over time, while in epithelial cells, synthesis of proteins *de novo* is limited to the initial hours following infection. It suggests that while the micro-environment changes in macrophages, it remains unaltered in epithelial cells during the first 24 h of infection (19).

Observation by electron microscopy suggests that *M. avium* can enter the intestinal mucosa by crossing either enterocytes or M cells (13). The same pathways of translocation may potentially occur in the respiratory tree where M cells are also present in large numbers (20). Recent studies, however, have established that *M. avium* uptake by the intestinal mucosa is primarily through enterocytes and not M cells (21), once more giving support to the hypothesis that the bacterium attempts to establish a niche before it can be detected by the immune system (figure 1)**.**

The efficiency of *M. avium* in binding and translocating across the intestinal mucosa of healthy mice varies according to the strain. While some strains (AIDS isolates) are very efficient, others do not achieve 10% of the level of infection (13). This experimental observation may be related to the fact that not all *M. avium* strains colonizing the intestinal tract of AIDS patients will ultimately cause disseminated disease. Non-AIDS strains of *M. avium* also seem to have impaired ability to invade intestinal epithelial cells (13).

Sangari and colleagues have recently shown that *M. avium enters intestinal* epithelial cells by the apical surface but not by the basolateral surface (22). Once inside the epithelial cells, *M. avium* acquires the invasive phenotype, being able to invade macrophages and other epithelial cells with remarkable efficiency (22). This aspect of *M. avium* pathogenesis probably is a common theme among intracellular pathogens which go through several stages inside the host in the course of infection. In addition, using time-lapse video microscopy, it is evident that uptake of *M. avium* by epithelial cells is not associated with ruffling as in the case of *Salmonella*, suggesting that

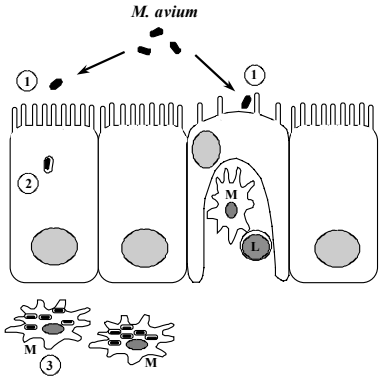

Figure 1: Current understanding of the *M. avium* interaction with the intestinal mucosa: (1) *M. avium* enters the mucosa by invading both enterocytes and M cells. It seems that there is a tropism for enterocytes as a preferable manner to invade; (2) once inside the host's cell, *M. avium* is found inside a vacuole; (3) the bacterium exits enterocytes by unknown mechanisms and is found in large numbers within macrophages in the lamina propria. M: macrophages, L: Lymphocytes.

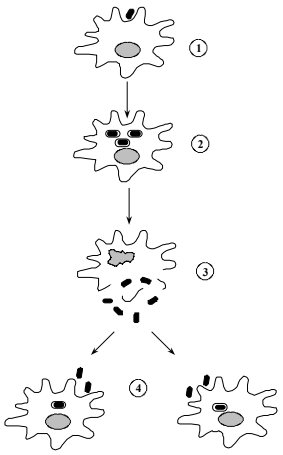

Figure 2: Scheme of stages of *M. avium* infection *in vivo*. (1) and (2) Bacterium is ingested by the first macrophage, soon after released from mucosal cells; (3) necrosis or apoptosis of macrophages release intracellular bacteria; (4) that can invade other macrophages by complement-receptor independent pathways.

invasion occurs by a pathway that does not require activation of the small GTP-binding protein cdc42 (23).

Because the mechanisms of host defense against *M. avium* probably are redundant, most individuals do not have disseminated disease, although they may have *M. avium* in intestinal lymph nodes.

3.2. Interaction with Phagocytes

Mycobacteria are facultative intracellular pathogens that characteristically reside within mononuclear phagocytes (24) During the last several years, we have begun to understand the extent to which extension intracellular bacteria have become masters at manipulating the structure and signaling pathways of the host cells for their own purposes in order to create a micro-environment that is suitable for multiplication and survival. A sophisticated manipulation of the host signaling pathways, with inhibition of some pathways in order to preclude hostile responses, or even activating other pathways to exploit them for invasion and/or survival.

The interaction of *M. avium* with macrophages is a typical example of a bacterium subverting host defenses on several levels. The first evidence of this phenomenon is the interaction of *M. avium* with receptors on the macrophage membrane. *M. avium* appears to recognize a number of receptors on the surface of macrophages and *M. avium* uptake by monocytes and macrophages has been associated with the presence of integrins, such as complement receptors (CR3, and CR4) (25, 26), the complement receptor CR1 (25), and vitronectin receptor (27), as well as with the mannose receptor (25).

Recent study has demonstrated that the expression of CR3 is upregulated upon *M. avium* binding to the vitronectin receptor (28). Mycobacteria also bind fibronectin by using related bacterial molecules, such as the antigen 85 complex (30-31 KDa proteins) (29) and the recently described fibronectin-attachment protein which has been characterized and shown to be highly conserved in both *M. leprae* and *M. tuberculosis* (9) The real importance of each one of the cited receptors for the uptake of *M. avium* by macrophages is currently unknown. It is tempting to hypothesize that both complement receptors and fibronectin receptors will be more important in the presence of serum whereas the other receptors could be relevant in the absence of serum. In addition, recently *M. avium* has been reported to recruit the complement fragment C2a to form a C3 convertase and generate active C3b in the absence of early activation components of the alternative or classical pathways (30). It seems that the predominant opsonin generated by this pathway is C3b instead of C3bi, as bacteria opsonized by this mechanism would bind primarily to CR1. Other receptors such as CD14 transferrin receptor and scavenger receptors have been described to mediate binding of *M. tuberculosis* (22, 31, 32) and *M. avium* to macrophages. There is evidence that *M. tuberculosis* (and maybe *M. avium*) binds to the lung surfactant protein and uses it as the link to be internalized by macrophages (33, 34). Their exact function is currently unknown. In the presence of serum, there is some evidence that fibronectin receptors may be involved in the uptake mechanism (figure 2).

Why *M. avium* (and *M. tuberculosis*) goes through the trouble of having a number of alternative ligands to specific membrane receptors on macrophage is currently unknown, but can be hypothesized as a strategy to be capable of entering macrophages in different sites and in different degrees of activation.

A common feature among all the receptors that mycobacteria has been shown to bind to is the fact that

they, in contrast to the Fc receptor, do not trigger oxygen burst in the cell (35). *Mycobacterium avium* can synthesize a superoxide dismutase of 23-25 KDa that can inactivate macrophage derived superoxide anion. In addition, recently, a homologous sequence to the OxyR gene of *E. coli* was found in the genome sequence of *M. leprae* (36). Apparently, a functional OxyR gene is also present in *M. avium*; however, further studies will be necessary to determine its importance in *M. avium* virulence (37). Indeed, the role of the respiratory burst of macrophages and whether suppressing it confers any advantage to mycobacteria is currently unknown. In vitro, *M. avium* strains vary regarding the resistance to superoxide anion and hydrogen peroxidase with some being resistant while others are susceptible (discussed below).

More recently, it has been shown that *M. avium* released from macrophages can invade other uninfected macrophages by complement receptor-independent mechanisms (32). This phenomenon may represent what in reality happens in vivo, since only a few mycobacteria are needed to establish infection in the host, but at later time points, hundreds of organisms can be seen in the site of infection. We can conclude that replicating mycobacteria ultimately leaves the infected macrophage either following apoptosis or necrosis and are capable of invading a neighboring macrophage. This concept has again been supported by an observation showing that in CD18 knockout mice, which do not express CR3 or CR4 receptors, the number of *M. avium* in deep tissue is comparable to the number of *M. avium* in tissue of wild type mice (38). Furthermore, the shape and size of tissue granulomas following infection were similar. Likeliwise, *M. avium* was present in macrophages in the spleen, ruling out uptake by different cells in P1 KO mice.

Mycobacterial-infected macrophages undergo apoptosis, that in vitro peaks at 3 to 5 days following infection (32). Whether apoptosis is triggered by the bacterium or by the host (as a mechanism of defense) is still undefined. A few studies have demonstrated that exogenous hydrogen peroxide-induced apoptosis of *M. avium* infected macrophages results in killing of intracellular bacteria (39). More recently, it was shown that the apoptosis of *M. avium*-infected macrophages is dependent on the secretion of TNF-α and Fas, and it is more often observed in activated macrophages than resting ones (40, 41).

3.3. Intracellular Lifestyle

Following phagocytosis by macrophages (and perhaps uptake by other cells), *M. avium* resides in a membrane-bound vacuole that does not acidify and never expresses proton pump ATPase (42, 43). Therefore, it is currently known that the pH of the *M. avium* endosome is approximately 6.5 to 6.8. Although there is some question about the possibility that *M. tuberculosis* exits the vacuole and ultimately lives in the cytoplasm (44), no information is currently available about *M. avium*. The intracellular environment certainly has great influence on *M. avium* resistance to killing mechanisms.

The mechanisms by which activated macrophages kill *M. avium* are poorly understood. A number of studies have tried to address this aspect of host response. The oxygen products (oxidative burst) were the first to be examined as a possible killing mechanism in phagocytes. Thus far, the conclusion that can be drawn from those studies is that *M. avium* strains vary in their susceptibility to oxygen radicals. While most of the AIDS isolates seem to be resistant to superoxide and hydrogen peroxide production (45, 46), some isolates have shown to be at least partially susceptible to reactive oxygen intermediates (45). In activated macrophages, which produce significantly more reactive oxygen intermediates, those strains can be significantly inhibited (47). However, as noted above, the majority of strains are not killed by superoxide anion or hydrogen peroxide (45, 46). *M. avium* expresses a superoxide dismutase (Mn-SOD) that is encoded by *sodA* (48). It has been shown that the bacterium secretes SOD, although the conditions or stimuli that trigger SOD production and release are not known (48). In addition, *M. avium* contains a functional *oxyR* gene (in contrast with *M. tuberculosis*), which is a regulator responsible for inducing the expression of several genes involved in the response to oxidative stresses. Whatever the mechanism(s), *M. avium* uptake by human and murine macrophages is associated with a significant suppression of superoxide anion production by the phagocytic cells (25, 32). Moreover, most *M. avium* strains are resistant to physiologic concentrations of hydrogen peroxide in vitro (45).

More recently, the attention has shifted to the possible role of reactive nitrogen intermediates (RNI) in the killing of *M. avium* by phagocytic cells. Although the majority of strains of *M. tuberculosis* seem to be susceptible to nitric oxide (49), the same does not appear to apply to *M. avium* strains. In fact, there is now evidence in vitro and in vivo that the great majority of AIDS isolates are resistant to nitric oxide (50-52)and that nitric oxide has no apparent role in the host defense against *M. avium* (52).

Bactericidal proteins produced by phagocytic cells have also been investigated as a potential mechanism of *M. avium* killing. Results of studies using rabbit defensins (small bactericidal proteins produced by rabbit neutrophils) supports the concept that *M. avium* may be susceptible to those proteins (53); however, no effect of human-derived bactericidal proteins has been demonstrated yet. Since all the evidence to date indicates that the *M. avium* phagosome never fuses with lysosomes (42, 43, 54-57), one wonders how bactericidal proteins (even if active against the bacterium) would come into contact with *M. avium*.

The expression of bactericidal activity in macrophages may depend on a successful orchestration of successive steps that start with the activation of the cell before contact with the bacterium is established, which would prevent *M. avium* from inhibiting acidification and fusion of the phagosome (42, 54). In this case, it is possible that some or all of the above discussed

mechanisms of killing may have a role in the elimination of the bacteria.

3.4. Interaction between *M. avium* and HIV-1

Studies of the pathogenesis of *M. avium* infection have always questioned the plausible impact of HIV-1 infection of macrophages and the interaction of HIV-1 with *M. avium* in the course of the infection. Early studies addressing this problem asked whether mononuclear phagocytes from AIDS patients had increased permissiveness to *M. avium* infection compared to mononuclear phagocytes from healthy individuals. Conflicting results were reported showing both that monocytes from AIDS patients had preserved function and responded to *M. avium* infection similar to monocytes from control individuals (58), as well as that cells from AIDS patients were permissive to intracellular growth of *M. avium* (59, 60). We have studied extensively over the years monocytes from AIDS patients and their ability to control *M. avium* infection as well as be stimulated with cytokines. Our results did not show a consistent pattern of response, but a large variation among the tested patients. Thus, while in some patients no significant difference could be detected between their cells and cells from a control individual, in others, permissiveness to *M. avium* growth and impaired response to stimulation with TNF-□ and GM-CSF were evident. Because only a small percentage of the circulating blood monocytes is infected with HIV-1 (61), we believed that it can explain the variation in results. In contrast, it may be very different in other tissues, where the majority of macrophages are infected with HIV-1 (62).

Work using dual infection of macrophages in vitro with HIV-1 and *M. avium*., however, showed a more distinct picture. While macrophages simultaneously infected with HIV-1 and *M. avium* did not show evidence of increased intracellular growth of *M. avium*, macrophages infected with HIV-1 prior to the infection with *M. avium* showed a consistent increase of intracellular replication of *M. avium* (63).

Because viral proteins are abundant in many tissues of AIDS patients, they may adversely affect macrophage function. This, in fact, has been shown by the work of Shiratsuchi and colleagues (64). These authors have demonstrated that HIV-1 envelop protein gp120 and core proteins p24 and gag5 when used to treat macrophages for 2 days prior to *M. avium* infection inhibited phagocytosis and enhanced intracellular growth of the bacteria. Production of cytokines such as IL-1, TNF-α and IL-6 were increased in macrophages infected with *M. avium* and exposed to gp120 in comparison with both macrophages exposed to gp120 and macrophages infected with *M. avium* (64). Thus, it seems clear that HIV-1 infection probably favors *M. avium* growth within macrophages and certainly has a negative impact on the outcome of the disseminated disease.

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