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MECHANISM OF *MYCOBACTERIUM AVIUM* COMPLEX PATHOGENESIS

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1. ABSTRACT

Mycobacterium avium complex (MAC) group of microorganisms are the most common opportunistic bacterial pathogens causing disseminated disease in HIV infected patients. These microorganisms are ubiquitous in nature, and are acquired by respiratory and oral routes. Pathogenesis of MAC depends on the ability of the organisms to colonize intestinal/respiratory mucosa, penetrate the protective barriers and resist intracellular killing by macrophages. Transient and reversible variation of colony morphology is one the characteristic feature of MAC that plays a significant role in the virulence and pathogenesis of these microorganisms. Isogenic colony variants of MAC differ in their virulence, susceptibility to antibiotics, stimulation of oxygen radicals and cytokines. The virulent smooth transparent colony variants are more frequently isolated from AIDS patients, more efficient in mucosal colonization, and adhere more efficiently to epithelial cells as compared to the less virulent smooth opaque variants. However, both the isogenic variants bind to the mucosal epithelial cells through the same multiple receptors. In addition, both the isogenic variants of MAC also bind to intestinal mucus through a single receptor. Study of the interaction of MAC with the host cells and characterization of MAC adhesins and host cell receptors facilitates the elucidation of the mechanisms involved in MAC pathogenesis.

2. INTRODUCTION

Infection may be considered as persistence and multiplication of a pathogen within the host, whereas significant damage to the host due to infection is considered as disease. A parasite to be successful, has to gain access to the host, colonize the host's tissue microenvironment and invade the tissues by overcoming protective mechanisms of the host. Normally, the host has innate as well as acquired mechanisms of prevention of colonization, by eliminating those microorganisms that gain access to different parts of the body. Gaining foot-hold in the host's tissues is an important prerequisite for invasion. To achieve these ends, the parasite devises various means to overcome protective barriers and undermines host's killing mechanisms. The parasite evades or subverts the host's surveillance mechanisms operating at various levels by producing different virulence factors. Opportunistic pathogens on the other hand, with inadequate virulence mechanisms, cause infections in immunodeficient individuals.

3. OPPORTUNISTIC INFECTIONS DUE TO *MYCOBACTERIUM AVIUM* COMPLEX

Mycobacterium avium complex group of organisms, consisting of *Mycobacterium avium* and *Mycobacterium intracellulare*, are ubiquitous in nature. These microorganisms are frequently isolated from water, plants and soil, and are distributed throughout the world. In the pre-HIV era, MAC were occasionally responsible for localized pulmonary infections in people with predisposing lung conditions like obstructive pulmonary disease, bronchiectasis, recurrent pneumonia, etc. (1). Advent of AIDS resulted in an increased incidence of MAC, and has been recognized as the most common opportunistic bacterial infections, causing disseminated disease in HIV infected individuals. Immunodeficiency following HIV infection predisposes to pulmonary or intestinal colonization of MAC (2). Subsequently, bacteremia and dissemination of the microorganisms to other organs, particularly the reticuloendothelial organs, ensues. MAC infected patients continue to excrete the organisms in the feces. MAC can be isolated from bronchial secretions and most of the organs at necropsy. A clear correlation has been shown between reduced $CD4^+$ T cell count and development of MAC disease (2,3). However, no other specific defect or attribute, immunological or otherwise, that contributes to the development of MAC disease has been identified. It is not known whether there is any defect in the protective barrier mechanism at the intestinal

Table 1. Immuno-patho-biological differences between the isogenic variants of MAC

FUNCTION	COLONY TYPE		REFERENCES
	ST	SO	
Virulence	++++	++	6,9,10
Intracellular growth	++++	+++	16,18
Stimulation of O_2 radicals/cytokines	++	++++	14,15,17
Adherence to epithelial cells	++++	++	8
Hydrophobicity	++++	++	8
Susceptibility to antibiotics	+	++	11
Cell wall sugars	++	++++	8

++++, high., +++, moderate., ++, low., +, least.

and respiratory mucosa enabling the microorganisms to colonize these sites, or a deficient bactericidal mechanism exist at the effector level of cellular immunity resulting in uncontrolled growth of MAC in the organs.

3.1. Virulence and Pathogenesis

MAC are the most versatile microorganisms. As an adaptation to their habitat, these organisms have evolved to grow at a wide range of pH and temperatures, and are innately resistant to most of the antibiotics and antimycobacterial drugs. MAC forms multiple colony types, which vary in pigmentation and possession of plasmids, and exist in several serotypes. The virulence factors and pathogenesis of these opportunistic pathogens has not yet been clearly understood. In order to investigate the problem in proper perspective, it is essential to recognize the attributes of the parasite and the host, and understand the nature of interaction from infection to colonization resulting in disseminated disease. The common portal of entry is considered to be the gastrointestinal route, in view of the facts that the organisms exist in water and soil, and large proportion of the MAC infected patients (81%) excrete the organisms in feces (4). Since MAC strains have been isolated from respiratory tract of AIDS patients (5), infection could be acquired through the respiratory route, albeit less frequently.

MAC strains exhibit a spectrum of virulence. AIDS-patients' isolates are more virulent than the environmental isolates (6,7). Of the several existing serotypes, serotypes 4,8 and 1 are commonly seen in HIV infected patients. A vast majority of MAC isolates from AIDS patients contain one or more plasmids. Attempts to draw a relation between serotype and virulence, or plasmid content and virulence are not convincing, since serotypes 4 and 8 are also seen in non-AIDS patients and plasmids are also seen in environmental isolates. In addition to the above attributes, MAC strains show isogenic variation, forming more than one colony morphology. The smooth transparent (ST) variants are the most frequent in clinical isolates while the smooth opaque (SO) and rough opaque (RO) forms are frequent with environmental isolates. Other colony forms like rough opaque (RO), intermediate and pin-point types are also encountered infrequently (8). Variation of colony morphology is transient and reversible. Prolonged incubation, and frequent subculture *in vitro* results in a change from ST to SO. Smooth transparent morphology is associated with increased virulence in chickens and mice (9, 10). It should be emphasized, however, that the virulence of MAC strains depends not only on formation of ST colony morphology but also on their stability (6). In addition to virulence, ST forms are more resistant to drugs than the SO forms (11), possibly by interfering with permeability (12, 13). Isogenic colony variants of MAC also differ in stimulation of oxygen radicals (14,15), intracellular multiplication (16) and stimulation of cytokines (17, 18) (table 1). The observed phenotypic changes are due to spontaneous changes in the genetic variation, which occur at relatively high frequencies in many species (19). The ability to vary immunogenicity of the important structural components plays an important role in the evolution of a successful pathogen. Such variations serve the organism as a means for escaping the immune response as well as for adapting to the microenvironment.

3.1.1. Mucosal Colonization

Since MAC infection occurs through gastrointestinal and respiratory routes, the microorganisms initially interact with respiratory and gastrointestinal mucosa and establish colonization. Before establishing contact with the mucosal epithelial cells, the microorganisms have to overcome entrapment in the mucus and elimination along with the secretions by ciliary and peristaltic movements. Adherence to the mucosal surface and particularly to the underlying epithelial cells plays an important role in colonization and subsequent translocation. In addition to colonization, adherence is a communication process with the cells. Binding to the surface molecules brings about transduction of various signals stimulating or altering different functions with or without invasion of the cells. Very limited knowledge is available regarding mycobacterial adherence and invasion, particularly of the mucosal epithelial cells with which they first encounter.

Development of beige mouse model by Gangadharam *et al* (20,21) which is used extensively for assessment of chemotherapeutic activity of new drugs also helped to understand the immuno-patho-biology of MAC disease to a certain extent. Beige mice can be easily infected by oral gavage resulting in colonization of the microorganisms in the intestine and excretion in the feces, followed by bacteremia and dissemination to other organs (21, 22). This mouse model offers unique advantage for the study of colonization and invasion. It has been shown that MAC bind to the epithelial cells (23,24), suggesting the presence of MAC receptors on the surface these cells. We have shown a significant association between the colony morphology and the ability of MAC to colonize the intestine in beige mice. After oral infection, ST colonial variants colonized the intestines and excreted continuously in the feces, whereas SO variants were eliminated quickly (25). The influence of normal flora of the intestine on MAC colonization and colony variation is not known. Since the patient's isolates form ST colonies and the environmental isolates form SO or RO colonies, it is not clear whether the occasional environmental microorganisms existing in ST form only cause the disease or the influence of the host's internal milieu causes the change in colony morphology from SO to ST forms thereby causing disease. So far, neither the virulence factors of MAC nor the specific immunological abnormalities

of the AIDS patients, making them vulnerable to MAC infections, have been identified. None of the existing immunological deficiencies in the AIDS patients could be directly attributed to their susceptibility to MAC disease.

3.1.2. Adherence to Epithelial Cells

MAC strains adhere and invade epithelial cells (22, 23). Log phase organisms were found to be more efficient than those in the stationary phase growth, and active participation of mammalian cells in the process of invasion has been observed (23). Smooth transparent variants were found to be more efficient in adherence to epithelial cells as compared to SO variants (8). A large body of data is available on the mechanism of entry of many enteric pathogens, but not much is known about the mechanism of adherence and invasion of MAC. In our studies, we have demonstrated that the virulent forms of MAC (ST colonial forms) were not only efficient in colonization of intestines (25) and adherence to epithelial cells (8), but were also more efficient in accumulation in Peyer's patches, as demonstrated in the mouse ligated ileal loop assay (author's unpublished results). It has been shown earlier that BCG and *M.tuberculosis* accumulate in Peyer's patches following oral infection (26). Enteric pathogens adopt different strategies for translocation across the intestinal wall. Increased accumulation of MAC in Peyer's patches clearly indicate utilization of M cell pathway for translocation of these microorganisms across intestine.

As far as virulence and colony morphology is concerned, there are remarkable similarities in the pathogenesis of *Streptococcus pneumoniae* and MAC. *Strep. pneumoniae* can exist as commensal microorganisms in healthy people, produces 3 types of colonial variants with the smooth transparent form being more virulent by virtue of its increased ability to colonize the nasopharynx and its propensity to bind to lung cells (27). Interestingly smooth transparent variants of *Strep. pneumoniae* bind to the platelet activating factor (PAF) receptor. Activation of lung cells with IL-1 and TNF-alpha results in increase expression of PAF receptor (resting lung cells do not express PAF receptors) thereby enhancing the binding specificities for virulent forms of *Strep. pneumoniae* (28). Bacterial adherence to epithelial cells has been shown to modulate secretion of cytokines. Adherence of uropathogenic *E. coli* to epithelial cells not only induces increased secretion of IL-6 and IL-8 but also upregulates the expression of adhesion molecules. Cytokines secreted by T cells and monocytes modify epithelial cytokine response to bacterial adherence (29). In this regard, it is pertinent to note that infection of monocytes with MAC causes increased release of several cytokines like IL-1, TNF-alpha and IL-6 (17, 18). Whether MAC infection also leads to increased release of cytokines from epithelial cells and the effect of these cytokines on the adherence and invasion of host cells needs to be investigated.

3.1.3. Mucus binding and Colonization

In addition to binding to intestinal epithelial cells, MAC adhesins bind to intestinal mucus (author's unpublished results), displaying another aspect of colonization. Mucus layer, containing mucins, acts as a protective layer for the epithelium. Mucins, the glycoproteins secreted by the goblet cells, act as substrate for the intestinal microorganisms. Binding of the microorganisms to mucus helps the colonization. On the other hand, mucus receptors may mimic epithelial receptors, preventing mucus bound microorganisms reaching the underlying epithelial cells. Enterotoxigenic *E.coli*, which induces diarrhea in pigs, express a plasmid which encodes the fimbrial antigen K88ab through which the organisms bind to the intestinal mucus and brush borders (30). Binding of mucins to *Entameba histolytica*, on the other hand, inhibits their adherence to Chinese hamster ovary cells (31). It remains to be determined whether binding of MAC to intestinal mucus interferes with their adherence to underlying epithelial cells. Since M cells have a thin covering of protective mucus, the chances of the microorganisms reaching these cells is high, consequently the microorganisms accumulate in Peyer's patches. In normal healthy individuals MAC may fail to reach the intestinal epithelial cells as the organisms are entrapped in the mucus and are eliminated. In AIDS patients, intestine is one of the primary organ of MAC involvement. Large number of MAC are seen in the mucosa, Peyer's patches, macrophages of the lamina propria and mesenteric lymph nodes in these patients (32,33). Mucosal erosion and diarrhea are common in AIDS patients. Loss of intestinal mucus due to increased bowel movement could enhance the chances of MAC reaching the epithelial cells thereby assisting invasion and dissemination.

3.1.4. Interaction with Macrophages and Lymphocytes

Once the microorganisms cross the epithelial barrier, the second line of innate defense consists of fixed and circulating mononuclear phagocytic cells of the host. Binding of the microorganisms to mononuclear phagocytes initiates phagocytosis, triggers release of cytokines and reactive oxygen radicals. The cytokines and other metabolites mediate inflammation- attract, retain and invoke the assistance of other immunocompetent cells in combating the invading microorganisms. Phagosomes containing the engulfed microorganisms fuse with lysosomal vesicles containing various enzymes and inhibitory agents exposing the phagocytosed microorganisms to deleterious action of these agents. However, MAC evade and/or subvert bactericidal mechanisms of the macrophages, multiply inside unhindered and eventually kill the phagocytic cells. The ability to withstand intracellular acidic pH, refractile nature of the cell wall and secretion of products which block fusion of lysosomes with phagosomes are some of the features of MAC which undermine the adverse intracellular milieu.

MAC multiplies efficiently in macrophages of genetically susceptible mice as compared with resistant ones, indicating a possible role of BCG gene in partial protection (34). MAC infection stimulates release of several cytokines from macrophages. The predominant cytokines are IL-1, TNF-alpha, IL-6, and IL-4 contributes to a lesser extent. Survival of infected macrophages correlates with the level of TNF-alpha and IL-6 production (35). Release of IL-1 is known to stimulate T-lymphocytes to produce IL-2, IFN-gamma and IL-4. IFN-gamma and TNF-alpha synergistically stimulate intracellular killing by macrophages. Iosgenic colony variants of MAC differ in the way they interact and stimulate macrophages. Smooth transparent variants are less efficient in stimulation of cytokines and oxygen radicals from macrophages (14,17). Further, ST variants were found to multiply efficiently inside macrophages

as compared to SO variants (16,18). Macrophages, activated by cytokines, such as IFN-gamma and TNF-alpha, display increased release of oxygen radicals upon stimulation and show increased bactericidal activity. TNF-alpha enhanced killing of the intracellular MAC in human macrophages (36) and mouse macrophages (37). While GM-CSF alone was found to be bacteriostatic, combination of GM-CSF and TNF-alpha had additive effect in macrophages (36). Bactericidal effect mediated by TNF-alpha has been attributed to the generation of reactive nitrogen intermediates (36). IL-2 has also been shown to augment intracellular killing of MAC by macrophages (38,39). IFN-gamma caused inhibition of intracellular growth of MAC in human macrophages (40), but not in mouse macrophages (37). While IFN-gamma and 1, 25 (OH2) vitamin D3 reduce bacterial growth in human macrophages (41) IL-1, IL-3, IL-6 and M-CSF are known to enhance the growth (41,42). Co-infection of macrophages with both MAC and HIV alters cytokine profile, cell viability, and causes increased multiplication of MAC as well as increased replication of HIV (43,44).

Another factor that seems to play a role in intracellular multiplication MAC is the intracellular iron level. Normal serum causes transient inhibition of growth of MAC in macrophages. Serum iron and transferrin levels are low in AIDS patients. Consequently, AIDS-patient's sera fail to inhibit the growth of MAC. However, intracellular iron storage levels are high in macrophages of these patients leading to increased multiplication of MAC. Further, addition of iron or iron-saturated transferrin to macrophage cultures enhances intracellular growth of MAC (45).Thus, intracellular sequestration of iron in macrophages interferes with mycobactericidal function of macrophages.

MAC infected patients show impaired T-cell response to specific antigens either by interfering with monocyte accessory cell functions (46,47) or by directly suppressing lymphocyte proliferation by the cell wall components, particularly the lipopeptide portion of the GPL (48,49,50). MAC infected macrophages secrete PGE2 (51), which is known to inhibit lymphocyte proliferation by interfering with lymphocyte mediated activation of macrophages through the release of IFN-gamma. Indomethacin, an inhibitor of PGE2, augments macrophage activation by IFN-gamma *in vitro* (42,52) and *in vivo* (51). In addition, MAC infection results in production of immunosuppressive macrophages which prevent generation of IL-2 reactive T cell population through the release of prostaglandins (53).

NK cells are known to play a role in protection against intracellular bacterial infections including MAC (54,55). *In vitro* studies have shown lysis of MAC infected macrophages by NK cells and large granular lymphocytes (54). NK cells are also known to produce cytokines, particularly IFN-gamma. It has been hypothesized that lysis of indolent MAC infected macrophages results in release of the organisms. However, the extent to which cytotoxic cells contribute to the protection against facultative intracellular mycobacteria as opposed to the pathogenesis is uncertain and needs further study.

3.2. MAC Adhesins and The Host Cell Receptors

Isogenic variants express several colony specific proteins (56,57). However, none of these proteins have been characterized, and their role in virulence and pathogenesis is not known. There are differences in glycolipid profile of the isogenic variants (8,58). Glycolipids of MAC have been shown to modulate lymphocyte (48,49,50) and macrophage functions (8). Nonetheless, ST variants do not display any unique glycoprotein contributing to the increased virulence (8). The isogenic variants also differ in the total cellular sugar content with SO forms having highest amount (8).

Adherence of MAC to mucosal surfaces involves multiple interactions, recognizing multiple mucosal receptors. By using labelled protein extracts of MAC, at lease six receptors were detected on HEp-2 cells. Three receptors were found on intestinal epithelial cells and one receptor was detected in intestinal mucus. Both ST and SO isogenic forms recognized the same receptor protein profile in all the mucosal extracts. All the MAC specific mucosal receptors were trypsin sensitive and periodate resistant indicating the protein or possibly glycoprotein nature of the receptors (author's unpublished results). MAC interact with the mucosal receptors through surface expressed adhesin(s) which are being identified. It is not known whether both the isogenic variants share the same adhesins.

Several mycobacterial species including *M. avium* have been shown to adhere to fibronectin (59). Fibronectin is involved in the adhesion of streptococci and staphylococci to host cells (60). Fibronectin binding protein has been purified and characterized. It contains 2 subunits of molecular mass of 55 and 22 kDa (61). Anti-55 kDa protein antibodies inhibit the attachment of viable BCG to fibronectin (62). Though these data indicate that fibronectin could play a potential role in virulence of mycobacteria, its role as a major virulence factor is debatable, since all the species of mycobacteria tested, including the non-pathogenic species, secrete fibronectin binding protein. In addition to fibronectin binding protein, MAC strains have been shown to bind to vitronectin on the surface of macrophages, and the binding was inhibited by specific antibodies (63). Vitronectin is an integrin which is present on the surface of cells including epithelial cells. Adhesion to these molecules could play a role in colonization of MAC. Integrins are a class of transmembrane surface receptors which are involved in cell-cell and cell-matrix binding. A large number of microorganisms bind and enter the cells by attaching to the integrin molecules (64,65). Many integrins bind to the ligands which share the common motif, tripeptide RGD (arg-gly-asp). RGD sequences have been recognized in bacterial proteins and have been shown to interact with the host receptors (65). However, MAC ligand that binds to vitronectin receptor lacks RGD sequences (63).

4. PERSPECTIVE

Intestinal or lung colonization precedes the development of MAC disease in AIDS patients. Even though normal healthy individuals are exposed to MAC, they rarely develop disease because the microorganisms fail to colonize, probably due to lack or inaccessibility of MAC binding receptors on the epithelial cells. It is quite possible that HIV infection directly or indirectly upregulates the expression of the surface receptors of intestinal and lung epithelial cells predisposing to MAC colonization. Structural changes in the

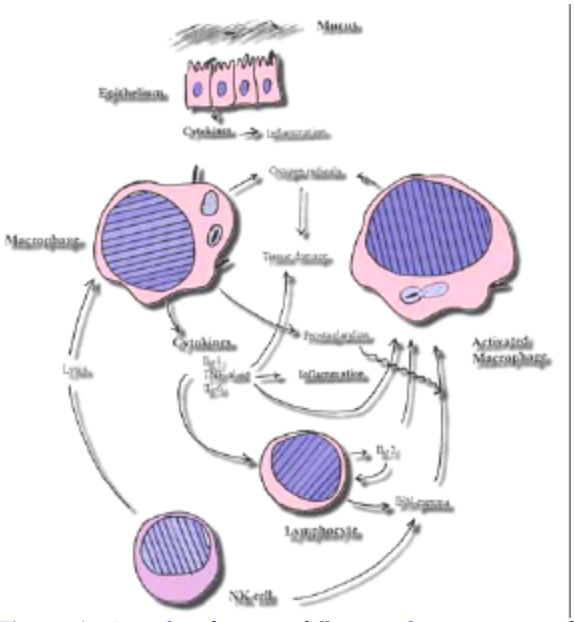

Figure 1. Cascade of events following the interaction of *Mycobacterium avium* with host cells.

microorganisms as a result of variation in colony morphology contributes to the virulence of the organisms significantly. Interaction of the microorganisms with different cells through specific receptors results in the release of cytokines and other mediators. The cascade of events (figure 1) initiated following the interaction of the microorganisms with epithelial and mononuclear phagocytic cells results in generation of macrophages endowed with increased killing of the invading microorganisms. These events direct and orchestrate the process of protection or pathogenesis. Macrophages are believed to kill the microorganisms through release of reactive oxygen radicals (0_2-,0H, H_20_2 and NO) as well as lysosomal enzymes. A delicate balance in the levels of cytokines and oxygen metabolites released during infection needs to be maintained in order to generate effective immunity. Identification and characterization of MAC adhesins and host cell receptors involved in generation of the protective response and understanding the mechanisms involved in the interaction of the MAC with the host will help in devising protective measures against MAC induced disease.

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