The pathogenesis of *Acanthamoeba* infections: current status and future implications

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SUMMARY

Acanthamoeba are opportunistic protozoan parasites that can cause painful, vision-threatening keratitis. However the pathogenesis and pathophysiology of *Acanthamoeba* keratitis remain incompletely understood. Most cases of *Acanthamoeba* keratitis develop as a result of poor hygiene in contact lens care but it is unclear how amoebae transmigrate from the environment into the cornea leading to inflammation, photophobia and blindness. *Acanthamoeba* keratitis has become increasingly important in the past few decades due to increasing populations of contact lens users. The mechanisms associated with the pathogenesis of *Acanthamoeba* are highly complex, depending on the virulence properties of the parasite, host susceptibility and the environmental conditions. Complete understanding of *Acanthamoeba* pathogenesis and its associated risks factors should allow us to design strategies for disease prevention and for the rational development of therapeutic interventions against these devastating infections.

Acanthamoeba keratitis has become a significant problem in recent years, especially in contact lens wearers exposed to contaminated water.

INTRODUCTION

Genus *Acanthamoeba* is a group of free-living protozoan that are widely distributed in the environment. For example, *Acanthamoeba* have been isolated from air, soil, freshwater, seawater, tap water, bottled mineral water, laboratory distilled water wash bottles, chlorinated swimming pools, sewage, food materials and considered as one of the most ubiquitous organisms (Khan and Paget, 2002; De Jonckheere, 1991; Kilvington and White, 1994; Ma *et al.,* 1990). This is further supported with recent findings that more than 80% individuals contain serum antibodies against *Acanthamoeba* antigens (Chappell *et al.,* 2001). The ubiquity of *Acanthamoeba* is a major contributing factor in *Acanthamoeba* infections as it provides them with a greater access to the susceptible hosts. *Acanthamoeba* are able to withstand these diverse environmental conditions by switching their phenotype. Under harsh environmental conditions such as deprivation of food, high temperature and high osmolarity, *Acanthamoeba* transform into a resistant cyst form, which is metabolically inactive and presents a major problem in therapy (Fig. 1). The reverse conditions would lead to the emergence of vegetative trophozoite form. *Acanthamoeba* trophozoite divide by binary fission and given the opportunity can cause serious human infections (Byers, 1979; Byers *et al.*, 1991; Ma *et al.*, 1990).

Genus *Acanthamoeba* consists of both virulent and avirulent strains. Recent advances in molecular techniques have lead to the classification of this genus into 13 different genotypes using rDNA sequence analyses i.e., T1 – T12 and T14 (Stothard *et al.,* 1998; Gast, 2001). The basis of this scheme is that each genotype exhibits at least 6% sequence difference from all the other

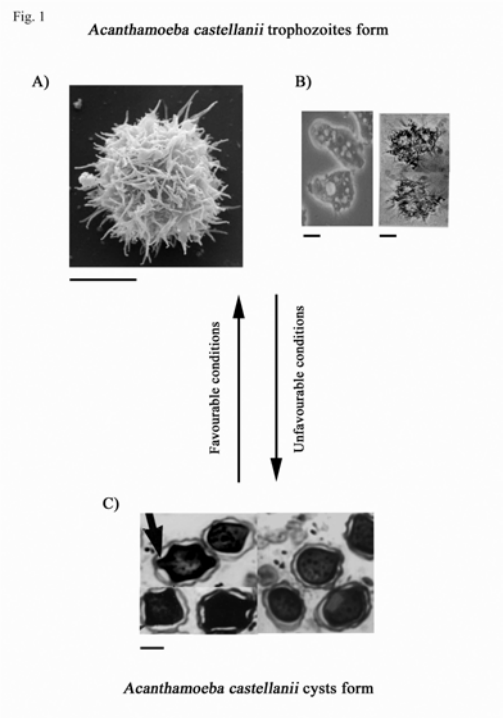


Figure 1. The life cycle of *Acanthamoeba castellanii*. A) Infective form of *A. castellanii*, also known as trophozoites, as observed under scanning electron microscope and **B)** under phase-contrast microscope. Under unfavorable conditions, trophozoites differentiate into cysts. **C)** Cysts form of *A. castellanii,* characterized by double wall as indicated by arrows. Bar = 5 μm (reprinted with permission from Microbial Pathogenesis).

genotypes (Gast *et al.,* 1996). However, sequence analyses of all keratitis isolates tested to date, revealed that keratitis-causing isolates belong to the genotypes, T3, T4, T6 and T11 (Stothard *et al.,* 1998; Khan *et al.,* 2002a; Walochnik *et al.,* 2000; Walochnik *et al.,* 2000a). Furthermore, it is shown that more than 95% *Acanthamoeba* isolates that produce keratitis belong to T4 genotype, suggesting that pathogenicity may be limited to a certain closely related genotypes (Stothard *et al.,* 1998; Khan *et al.,* 2002a; Walochnik *et al.,* 2000).

For example, Booton *et al.,* (2002), tested 13 *Acanthamoeba*, isolated from clinical samples, contact lenses, lens cases or lens case solutions in Hong Kong. The results revealed that 12 belong to T4 genotype and one T3 isolate. Again, in a recent study, De Jonckheere (2003), has determined T4 as the sole keratitis-producing genotype in all nine *Acanthamoeba* keratitis cases in Belgium. Although the rDNA sequence information of an organism is not a measure of isolate's pathogenicity, the above findings conclusively indicate that sequence information can be used to indicate the pathogenic potential of a given isolate, which should be further verified using *in vitro* and *in vivo* models. Overall, these studies have shown that T4 is the major keratitis-producing genotype. However, whether predominance of T4 isolates in keratitis is due to greater virulence or greater prevalence remains to be determined.

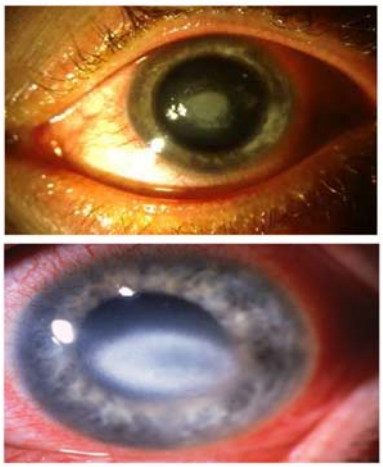


Figure 2. *Acanthamoeba* infected eye exhibiting the severity of the disease. Note the ulcerated epithelium and stromal infiltration exhibiting corneal opacity in acute *Acanthamoeba* keratitis (reprinted with permission from Microbial Pathogenesis).

Given access and the susceptible hosts, pathogenic *Acanthamoeba* can cause blinding keratitis (Fig. 2). *Acanthamoeba* keratitis is initiated by direct exposure of the eyes to contaminated water and more frequently with poor hygiene in the contact lens use. The latter is facilitated with washing contact lenses in tap water, home-made saline as well as contact lens wear during swimming. Contact lens wear for extended periods of time and chlorine-based cleaning solutions are additional risk factors associated with *Acanthamoeba* keratitis. *Acanthamoeba* keratitis is characterized by severe pain due to radial neuritis and inflammation with redness and photophobia. If not diagnosed early and treated aggressively, the corneal epithelium becomes ulcerated with stromal infiltration (Fig. 2) leading to perforation, ring infiltrate and, finally, loss of vision. To date, there have been more than 1350 estimated cases of *Acanthamoeba* keratitis reported worldwide with a continuous rise due to an increasing population of contact lens wearers (Martinez, 2001). Other infections due to *Acanthamoeba* include granulomatous amoebic encephalitis (GAE) that often results in death. GAE is thought to be initiated by entry of amoebae through lower respiratory tract or skin lesions, followed by hematogenous spread. Although not proven clinically, it is likely that circulating amoebae cross the blood-brain barrier to gain entry into the central nervous system (CNS) to produce disease. GAE is characterized by subacute or chronic granulomatous encephalitis with clinical features such as, stiff neck, headache, fever, nausea and seizures. These conditions could last for several months, and mostly affect immunocompromised patients (Ma *et al.,* 1990).

Table 1. Risk factors associated with *Acanthamoeba* keratitis

No.	Risk factors
1	Handling of contact lenses (CL) with unclean hands
2	Washing CL with home-made saline / tap water
3	CL wear for more than recommended times
4	CL wear during swimming
5	Washing eye/swimming with corneal trauma – splashing eyes with contaminated water
6	Reusing CL without proper cleaning
7	Incubating CL in disinfectants for less than recommended times
8	Chlorine-based disinfectants are less effective in killing *Acanthamoeba*
Recommendations –	
Combination of disinfectants, which can prevent biofilm formation in contact-lens storage case and effectively kill *Acanthamoeba*	
Improving personal hygiene and dealing with eye with gentle care	

Despite the increasing importance of *Acanthamoeba* infections and their devastating consequences, the precise mechanisms associated with the pathogenesis of *Acanthamoeba* remain unclear. Understanding the molecular basis of *Acanthamoeba* pathogenicity is crucial for the development of therapeutic interventions. It is well-established that pathogenicity is a complex process that involves multiple factors, both from the parasite side and the host side, and there is no single determinant that causes or permits these parasites to produce human diseases. This review describes some of the determinants, which directly or indirectly contribute to *Acanthamoeba* keratitis and may also prove relevant in GAE. This by no means should be considered as a complete list, and undoubtedly, further research will continue to identify factors responsible for *Acanthamoeba* pathogenesis.

A) Direct virulence factors

1. Contact-dependent mechanisms

1a. Adhesion

Adherence is an important microbial property required for colonization, persistence and a key step in the infection. Thus anti-adhesion therapies provide attractive approaches to prevent many infectious diseases. Adhesion in *Acanthamoeba* is known to be a prerequisite in its pathogenesis and is mediated by an adhesin-receptor fashion. *Acanthamoeba* uses adhesion to host cells both as an initial step before proceeding to the deeper tissue to produce keratitis as well as to avoid being washed out during the onset of the disease. It is well-recognized that binding of pathogenic *Acanthamoeba* to corneal epithelial cells is mediated by amoeba lectin i.e., a 130 KD subunit of ~400 KD oligomer (composed of at least three 130 KD subunits). MBP (~833aa) consists of a N-terminus domain (residues 1-21 that codes for cleavable signal peptide), a large extracellular domain, a transmembrane region and a C-terminus cytoplasmic domain. The extracellular domain contains five putative N-glycosylation sites and three O-glycosylation sites (Garate et al., 2004). Adhesion of *Acanthamoeba* to host cells can be inhibited in the presence of exogenous α-mannose (Morton *et al.,* 1991; Yang *et al.,* 1997). Recent studies have shown that mannose-binding protein mediates

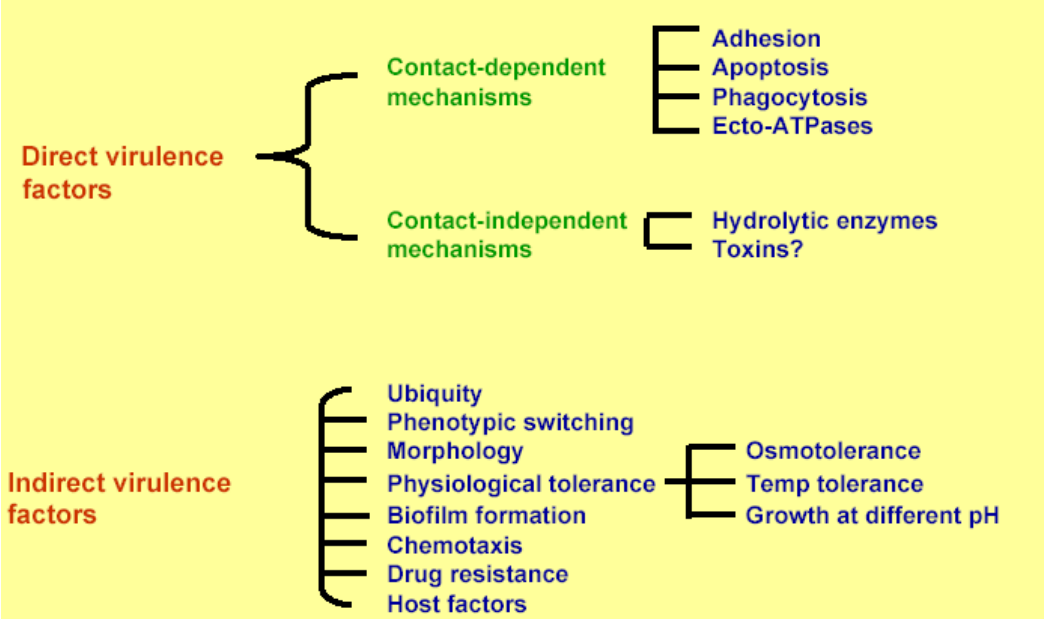
Acanthamoeba binding to human brain microvascular endothelial cells, which may lead to the transmigration of amoebae to the central nervous system (Alsam *et al.,* 2003), an important step in the development of fatal GAE. These findings further indicate that mannose-binding protein is an important *Acanthamoeba* adhesin responsible for primary interactions with the host cells. Similar concepts have been established in other protozoan parasites such as *Entamoeba histolytica*, which is known to bind and lyse the host cells using its galactose-binding protein (reviewed in McCoy *et al.,* 1994). Moreover, *E. histolytica*-produced cytotoxicity on host cells is abolished in the presence of exogenous galactose suggesting that it is a contact-dependent mechanism. In an attempt to determine the role of binding in the pathogenesis of *Acanthamoeba*, cytotoxicity assays were performed in the presence of exogenous α-mannose, however results are somewhat controversial. Using *in vitro* assays, Cao *et al.,* (1998), showed that inhibition of amoeba binding to corneal epithelial cells using exogenous mannose resulted in the loss of the ability of *Acanthamoeba* to produce cytotoxicity on corneal epithelial cells. In contrast, Leher *et al.,* (1998) showed that addition of exogenous mannose exacerbated the ability of *Acanthamoeba* to produce cytotoxicity on corneal epithelial cells. In support of this, it was further shown that binding of *Acanthamoeba* to mannose residues resulted in increased secretion of serine proteases, which are major determinants in the host cell damage (Leher *et al.,* 1998; Khan *et al.*, unpublished data). These studies have indicated that *Acanthamoeba* binding to host cells is mediated by mannose-binding protein, however, binding leads to multiple secondary processes such as phagocytosis (Khan, 2001), and secretion of proteases (Cao *et al.,* 1998; Leher *et al.,* 1998; Hurt *et al.,* 2003), which may play a direct role in the pathology of the disease. However, inhibiting one process, such as binding using α-mannose, leads to increase in protease secretion that leads to direct cellular and tissue damage. These findings suggest that engagement of mannose-binding protein leads to intracellular signal transduction pathways, which stimulate the secretion of protease(s). At present, it is unclear whether mannose-binding protein-mediated protease secretion involves transcription and translation of protease(s) or simply regulated at the secretory level. Ideally, inhibition of binding without the correct engagement of the mannose-binding protein may help us understand the role of binding in *Acanthamoeba* pathogenesis as well as its use in the development of therapeutic interventions. For example, it is shown that anti-Acanthamoebic IgA antibodies inhibit their binding to host cells as well as block the secretion of cytotoxic substances and play a crucial role in the ultimate macrophage-mediated complement lysis and provide protection against *Acanthamoeba* keratitis *in vivo* model (Ferrante 1991; Cursons *et al.,* 1980; Leher *et al.*, 1999). However, reports are beginning to emerge indicating that pathogenic *Acanthamoeba* can resist macrophage-mediated complement lysis (Toney and Marciano-Cabral 1998). In support, Walochnik *et al.,* (2001) have shown that pathogenic *Acanthamoeba* bind C1q, component of complement pathway, which under normal conditions provide basis for opsonin and phagocytosis process. Additionally, Niederkorn *et al.,* (1999), have shown that pathogenic *Acanthamoeba* can degrade IgG and IgA antibodies. These studies have

suggested that pathogenic *Acanthamoeba* have potential to interfere with the immune system, which may provide a basis to evade host defenses, further indicating the need for developing therapeutic interventions.

As described above, the genus *Acanthamoeba* has been recently classified into 13 different genotypes (T1-T12 & T14). In an attempt to correlate pathogenicity with a given genotype, we tested *Acanthamoeba* isolates belonging to genotypes T1, T2, T3, T4, T7 and T11 for their ability to bind to corneal epithelial cells and brain microvascular endothelial cells. We observed that isolates belonging to genotypes T1, T3, T4 and T11 exhibited higher binding to the host cells, while T2 isolates, exhibited less. Isolates belonging to T7 genotype exhibited minimal binding. More importantly, differences between T1, T3, T4, T11 and T2, T7 isolates, as well as, between T2 and T7 were significant ($P<0.05$). Moreover, *Acanthamoeba* isolates, which exhibited higher binding, showed deleterious host cell cytotoxicity using *in vitro* assays. However, the reason for these differences within the genus *Acanthamoeba* and whether these differences are genotype-dependent or host species-dependent remains unclear. Alternatively, it is possible that mannose-binding protein is absent or antigenically distinct in different genotypes of *Acanthamoeba* (previously suggested by Kennett *et al.,* 1999). Overall, these data suggest that mannose-binding protein is an important *Acanthamoeba* adhesin and potential target for future research, which will determine its precise role in the pathogenesis of *Acanthamoeba* infections *in vivo*.

1b. Phagocytosis

As described above, binding of *Acanthamoeba* to host cells stimulate secondary processes such as phagocytosis. Phagocytosis is a process by which *Acanthamoeba* "bites-off" or engulf host cells and food particles. Phagocytosis in *Acanthamoeba* has been shown using a variety of particles including latex beads (Avery *et al.,* 1995; Korn, 1974), bacteria (Preston and King, 1984) and yeast (Bowers and Olszewski, 1983; Allen and Dawidowicz, 1990). However, to fully establish the role of phagocytosis in the pathogenesis of *Acanthamoeba* keratitis, we have recently shown that *Acanthamoeba* phagocytose primary corneal epithelial cells (Khan, 2001). We further showed that engulfment of corneal epithelial cells by *Acanthamoeba* requires cytoskeletal rearrangements and is mediated by amoebastomes, present on the surface of *Acanthamoeba* suggesting that phagocytosis may play a major role in the pathogenesis of *Acanthamoeba* infections (Fig. 3). In support, it is shown that actin polymerization inhibitor, cytochalasin D, blocks *Acanthamoeba*-mediated host cell cytotoxicity (Taylor *et al.,* 1995). However, specific molecular interactions and the underlying intracellular signaling pathways leading to cytoskeletal rearrangements and uptake remain unclear. Similar structures have been observed and implicated in the pathogenesis of other protozoan parasites such as *Naegleria* (John *et al.,* 1985) and *Entamoeba histolytica* (Gonzalez-Robles and Martinez-Palomo, 1983; Lushbaugh *et al.,* 1978; Martinez-Palomo and Martinez-Baez, 1983). Overall, these findings suggest that phagocytosis is an important virulence property of *Acanthamoeba*, which may play a significant role in host cell and tissue damage.



Factors contributing to the pathogenesis of *Acanthamoeba* infections.

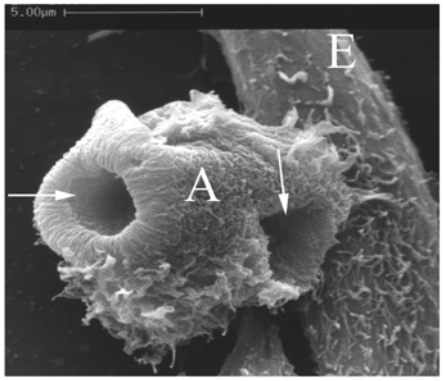


Figure 3. *Acanthamoeba* phagocytose corneal epithelial cells. *Acanthamoeba* incubated with corneal epithelial cells exhibited the presence of amoebastomes within 30 minutes of incubation as indicated by arrows. These structures are known be involved in the phagocytosis of *Acanthamoeba*. A is amoeba, E is corneal epithelial cell. (Published with permission from Current Microbiology).

The host cell response to *Acanthamoeba* challenge is not clear. Recent studies have shown that *Acanthamoeba* induces cell cycle arrest in the human corneal epithelial cells and human brain microvascular endothelial cells. This is shown by observations that *Acanthamoeba* inhibits expression of genes including cyclins F, G1 and cyclin dependent kinase 6 that encode proteins important for cell cycle progression and by upregulating expression of genes such as GADD45A and p130 Rb, associated with cell cycle arrest (Sissons *et al.,* 2004). This is further confirmed at the protein level by studying protein retinoblastoma (pRb) phosphorylations. Retinoblastoma is a potent inhibitor of G1/S cell cycle progression, however its function is inhibited upon phosphorylation, allowing progression into the S-phase. Our studies revealed that *Acanthamoeba* abolished pRb phosphorylations leading to cell cycle arrest at G1-to-S transition. Taken together, these studies have shown, for the first time that *Acanthamoeba* inhibits host cell cycle at the transcriptional level as well as by

modulating pRb phosphorylations using host cell signalling mechanisms (Sissons *et al.,* 2004). Other studies have shown that *Acanthamoeba* produces host cell DNA fragmentation, chromatin condensation and membrane blebbing, all well-known markers of apoptosis in neuroblastoma cells, representing yet another mechanism of host cell death (Alizadeh *et al.* 1994; Dove Pettit *et al.* 1996). However, whether cell cycle arrest leading to apoptosis and phagocytosis have independent roles in the pathogenesis of *Acanthamoeba* infections or apoptosis is a primary process, which is stimulated by initial binding of parasites with the host cells and leads to secondary events, such as phagocytosis, remains to be determined.

Ecto-ATPase activities

Ecto-ATPases are glycoproteins present in the plasma membranes with their active sites facing the external environment, which suggest that ecto-ATPases may be involved in surface membrane interactions between parasites and their host cells. Ecto-ATPases in protozoa are thought to be important for protection from the cytolytic effects of ATP, cellular adhesion, regulation of ecto-kinase substrate concentration, termination of purinergic signalling and involvement in signal transduction indicating that ecto-ATPases may play important roles in the pathogenesis of protozoan pathogens (Meyer-Fernandes *et al.,* 1997; Berredo-Pinho *et al.,* 2001; Plesner, 1995). Using *in vitro* assays, it is recently shown that *Acanthamoeba* isolates exhibit ecto-ATPase activities (Sissons *et al.,* 2004). These findings are of particular importance as previous studies by Mattana *et al*., (25) demonstrated that ADP release from *A. castellanii* play an important role in its contact-independent cytotoxicity, as both purified ADP and *A. castellanii* culture supernatant exhibited increased levels of intracellular calcium, which subsequently leads to apoptosis in Wish cells. This is further supported by Sissons *et al.,* (2004), who were able to inhibit *Acanthamoeba*-mediated human corneal epithelial cell cytotoxicity using ecto-ATPase inhibitor, suramin (P2 receptor antagonist), clearly demonstrating that ecto-ATPases play an important role in the pathogenesis of *Acanthamoeba*. In addition, exogenous α-mannose significantly increased ecto-ATPase activities of *Acanthamoeba* ($P<0.05$), while other sugars had no effect suggesting ecto-ATPases may well be associated with the mannose-binding protein (Sissons *et al.,* 2004). This concept is supported with the previous findings that exogenous galactose stimulate ecto-ATPase activities of *Entamoeba histolytica*, which posses galactose-binding protein (Barros *et al.,* 2000). These findings together with our observations suggest that adhesion lectins may be associated with ecto-ATPase activities. Future studies will precisely determine how ecto-ATPases are associated with the mannose-binding protein, which may help us identify potential targets to intervene these serious infections.

2. Contact-independent mechanisms

2a. Extracellular proteases

Proteolytic enzymes or proteases are a group of enzymes that catalyse the degradation of peptide bonds. Proteases are major pathogenicity determinants in parasitic protozoa, and are directly involved in cell and tissue invasion and damage. Proteases have been implicated in the pathogenesis of various protozoan parasites such as *Entamoeba* (Keene *et al.,* 1986; Keene *et al.,* 1990), *Trichomonas* (Arroyo *et al.,* 1989), *Leishmania* (Alfieri *et al.,* 1989; Bouvier *et al.,* 1987),

Trypanosome (Bontempi *et al.,* 1989; Kornblatt *et al.,* 1992; Souto-Padron *et al.,* 1990), and *Plasmodium*.(Bernard *et al.,* 1987; Chan *et al.,* 1974; Dluzewski *et al.,* 1986). A number of studies have shown the presence of extracellular proteases in pathogenic *Acanthamoeba*. For example, Hadas and Mazur (1993), demonstrated the presence of a 35 and a 65 KD serine proteases from eight species of *Acanthamoeba*. Later studies revealed the presence of 43, 59, 70 and 100-130 KD cysteine proteases and 33, 42, 47, 60, 75, 100 and 133 KD serine proteases in *Acanthamoeba* (Alfieri *et al.,* 2000; Cho *et al.,* 2000; Kong *et al.,* 2000; Kim *et al.,* 2003; Leher *et al.,* 1998). Other studies have shown the presence of additional 12, 107 and 230 KD serine proteases (Cao *et al.,* 1998; Khan *et al.,* 2000; Na *et al.,* 2001), as well as other hydrolytic enzymes such as elastase (Ferrante and Bates, 1988), phospholipase A (Cursons & Brown, 1978; Mishra *et al.,* 1985; Victoria & Korn, 1975) and there is some indication of metalloprotease activities in *Acanthamoeba* (Mitro *et al.,* 1994). The diverse nature of results in these studies may well be due to their complex biology or the differences in *Acanthamoeba* strains. Alternatively, parasites grown under various conditions using different nutritional supplements may exhibit diverse protease activities. However, to date, there is a common consensus on the importance of serine proteases in the pathogenesis of *Acanthamoeba*. This is supported by recent findings, which showed a direct cytotoxic role of extracellular serine proteases in host cell cytotoxicity *in vivo* assays (He *et al.,* 1990; Na *et al.,* 2001). It was also shown that inhibition of proteases using phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor) abolished host cell cytotoxicity of corneal epithelial cells clearly suggesting that serine proteases play a crucial role in the pathogenesis of *Acanthamoeba* keratitis (Leher *et al.,* 1998). Furthermore, pathogenic *Acanthamoeba* exhibited higher serine protease activities as compared to non-pathogens (Khan *et al.,* 2001). *Acanthamoeba* proteases mediate cytotoxicity by affecting host cell cytoskeleton and require increase in cytosolic free-calcium levels (Mattana *et al.,* 1997; Taylor *et al.,* 1995). In addition, extracellular proteases of *Acanthamoeba* appear to stimulate apoptosis in neuroblastoma cells (Alizadeh *et al.,* 1994). Whatever the mechanisms of host cell death, the precise target(s) of proteases remain unknown. In addition, it is unclear whether host cell death requires a single or multiple proteases. If they are multiple, then, how are their activities coordinated or are they acting independently of each other? These questions need to be addressed before we can begin to fully appreciate the complexity of the pathogenic mechanisms of *Acanthamoeba* infections. Recent studies have shown that human corneal epithelial cells express protease-activated receptors (PARs) (Lang *et al.,* 2003). PARs are G-protein-coupled receptors that are expressed on various cell types and are known to mediate cellular responses to injury, inflammation, or infection (Vu *et al.,* 1991). PARs are activated upon proteolytic cleavage of their N-terminus by serine proteases. This cleavage exposes the ligand domain of the receptor that binds to and activate the cleaved receptor (Lang *et al.,* 2003). To date, four members of PARs family have been identified, PAR-1, PAR-2, PAR-3 and PAR-4. Among these, PAR-1 has been implicated as a key mediators in cellular functions and activated by PAR-1 agonists, thrombin as well as by serine proteases, and PAR-2 are activated by trypsin-like

serine proteases (Cirino *et al.,* 1996; Grandaliano *et al.,* 1994; Naldini *et al.,* 1993). Of interest, PAR-1, PAR-2 and PAR-3 are all expressed on human corneal epithelial cells (Lang *et al.,* 2003) suggesting that amoeba serine proteases may act in a receptor-dependent fashion to produce host cell damage. Identification of the receptor(s) for amoeba proteases will lead to dissect the precise host intracellular signaling pathways, which results in cellular and tissue damage. Studies in the understanding of the role of proteases and their precise mechanisms will undoubtedly identify targets for therapeutic interventions.

B) Indirect virulence factors

As indicated above, the ability of *Acanthamoeba* to produce human diseases is a multifactorial process and is dependent on its ability to survive outside its mammalian host for variable periods of time and under diverse environmental conditions, such as, high osmolarity and varying temperatures on the surface of cornea. In this section, I will describe the role of environmental as well as host factors, which may play key roles in determining the successful transmission of these pathogens to a susceptible host.

1. Morphology

Infective forms of *Acanthamoeba* or trophozoites do not have a distinct morphology, however, they do possess spine like structures known as acanthopodia on their surface, which may play a key role in the pathogenesis of *Acanthamoeba* infections (Khan, 2001). Previously, we have shown that binding of pathogenic *Acanthamoeba* to corneal epithelial cells is mediated by acanthopodia (Khan, 2001). We further showed that *Acanthamoeba* which lack acanthopodia (non-pathogens) did not exhibit binding to corneal epithelial cells suggesting that these structures may work as a protraction to make initial contact with the host cells (Fig. 4). It will not be surprising to find that mannose-binding protein, which is involved in binding of amoebae with host cells is localized on acanthopodia, however, this has not been determined. Overall, these studies have suggested that acanthopodia represent a virulence trait (Fig. 4). However, more extensive studies are needed to fully establish the role of acanthopodia and other morphological traits in the pathogenesis of *Acanthamoeba*.

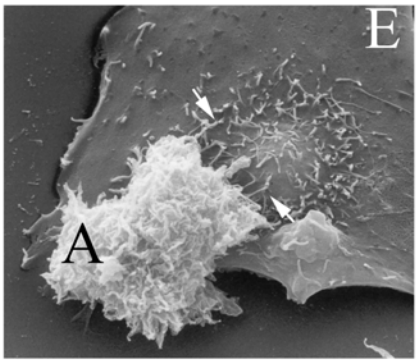


Figure 4. Binding of *Acanthamoeba* to corneal epithelial cells is mediated by acanthopodia. *Acanthamoeba castellanii* were incubated with corneal epithelial cells, and observed under scanning electron microscope. Note that parasites were able to exhibit binding to the host cells and binding was mediated by acanthopodia. A is amoeba, E is corneal epithelial cell. Bar = 10 μm (reprinted with permission from Microbial Pathogenesis).

2. Temperature tolerance and osmotolerance

Upon contact with tear film and corneal epithelial cells, *Acanthamoeba* are exposed to high osmolarity (for example salinity in tears) as well as high temperatures. For successful transmission, amoebae must withstand these burdens and exhibit growth.

Growth at high temperature and high osmolarity are known to be the hallmark of pathogenic *Acanthamoeba* (De Jonckheere, 1983; Ficker, 1988; Khan *et al.,* 2001a; Khan *et al.,* 2002; Walochnik *et al.,* 2000a). These studies have shown that the ability of *Acanthamoeba* to grow at high temperature and high osmolarity can be directly correlated with the pathogenicity of *Acanthamoeba* isolates. However, the precise mechanisms of pathogenic *Acanthamoeba* to adapt to higher temperatures and maintain metabolic activities remain entirely unknown. For example, temperature tolerance studies in *Candida neoformans* have identified Ca^{2+}-dependent protein phosphatase calcineurin to be required for its growth at 37°C (Odom *et al.,* 1997; Odom *et al.,* 1997a). Furthermore these studies showed *C. neoformans* strains in which calcineurin gene has been disrupted are avirulent in an *in vivo* model of cryptococcal meningitis. These studies might serve as a basis and a stimulus for research in the pathogenesis of *Acanthamoeba* infections. Overall, these simple growth assays have been used as markers for the differentiation of pathogenic and non-pathogenic *Acanthamoeba* isolates.

3. Growth at different pH

Pathogenic *Acanthamoeba* can grow both at acidic and basic pH, which suggests its ability to colonize several niches. *Acanthamoeba* are known to grow at pH ranging from 4 – 12 (Ficker, 1988; Khan *et al.,* unpublished data). However, the clinical significance of the ability of *Acanthamoeba* to exhibit growth at different pH is not known. For example, in *Candida albicans*, the ability to grow at diverse pH is crucial for its virulence (Davis *et al.,* 2000). Further studies identified two pH-regulated genes, *PHR1* (expressed at neutral and basic pH) and *PHR2* (expressed at acid pH). Deletion of *PHR2* resulted in loss of virulence and deletion of PHR1 resulted in reduced virulence in a systemic model (De Bernardis *et al.,* 1998). Again, similar studies will form a basis and provide a model to determine whether growth of *Acanthamoeba* at different pH plays a role in the pathogenesis of *Acanthamoeba* infections.

4. Phenotypic switching

Phenotypic switching is the ability of *Acanthamoeba* to differentiate into a morphologically distinct dormant cyst form or a vegetative trophozoite form. This is a reversible change and is dependent on environmental conditions (Fig. 1). Cysts are resistant to various antimicrobial agents and adverse conditions, thus, presenting a problem in chemotherapy because this may lead to recurrence of the disease. In addition, cysts can survive harsh environmental conditions, such as high temperatures and desiccation and they can be airborne (Byers, 1979; Cordingley *et al.*, 1996; Turner *et al.*, 2000; Weisman, 1976). Furthermore, *Acanthamoeba* cysts can survive up to several years while maintaining their pathogenicity (Mazur *et al.*, 1995). These characteristics suggest that the primary functions of cysts may lie in withstanding adverse conditions and in the spreading of amoebae throughout the environment. In addition, this may represent the ability of *Acanthamoeba* to alternate expression of surface proteins/glycoproteins, which may help evade immune surveillance in addition to changing environments. Most of these variations can be reversible and lead to the expression of predefined traits. At present it is not clear whether other mechanisms such as antigenic variation exists and their relevance in phenotypic switching. However, future

studies will address these issues. Overall, phenotypic switching represents a major factor in the transmission of *Acanthamoeba* infections, however, the underlying molecular mechanisms in these processes remain to be determined.

5. Drug resistance

Current treatment for *Acanthamoeba* keratitis involves topical application of mixture of drugs including chlorhexidine, polyhexamethylene biguanide (PHMB), neomycin and propamidine isethionate. These drugs have been shown to be most effective in killing *Acanthamoeba* trophozoites (Cohen *et al.*, 1987; Hay *et al.*, 1994; Larkin *et al.*, 1992; Lim *et al.*, 2000; Lloyd *et al.,* 2001; Moore and McCulley 1989; Murdoch *et al.,* 1998; Russell and Chopra, 1996; Seal *et al.,* 1995; Turner *et al.,* 2000; Wright *et al.,* 1985). Both chlorhexidine diacetate and PHMB are "membrane-acting" cationic biocides. At alkaline pH, surface proteins of *Acanthamoeba* are negatively charged interacting rapidly with these cationic biocides inducing structural and permeability changes in cell membrane leading to leakage of ions, water and other cytoplasmic components resulting in cellular damage (Perrine *et al.,* 1995). Other drugs are propamidine isethionate belonging to diamidine family and are effective DNA synthesis inhibitor (Duguid *et al.,* 1997). Of concern, several studies have recently shown the increasing resistance of Acanthamoebae to antimicrobial chemotherapy, however, the mechanisms of drug resistance in *Acanthamoeba* remain incompletely understood (Ficker *et al.,* 1990; Larkin *et al.,* 1992; Lim *et al.,* 2000; Lloyd *et al.,* 2001; Murdoch *et al.,* 1998). One intriguing report was made by Ficker *et al.,* (1990), who observed the development of propamidine resistance during the course of therapy for *Acanthamoeba* keratitis, which lead to recurrence of the infection. This may be due to the fact that propamidine isethionate belong to diamidine family and are effective DNA synthesis inhibitor (Johnson & Thomas, 2002), but may not be potent against cyst forms of *Acanthamoeba* at recommended concentrations, due to their reproductive and metabolic inactivity. Also, *Acanthamoeba* cysts are double-walled consisting of an inner endocyst and an outer ectocyst (composed of 33% protein, 4 – 6% lipids and 35% carbohydrates, mostly cellulose), which may also provide a physical barrier against various chemotherapeutic agents (Neff & Neff, 1969; Turner *et al.,* 2000). Additional factors may involve selection under continuous drug pressure with genetic basis such as point mutations or transferable genes and/or variations in metabolic activities. Precise understanding of these mechanisms is crucial for the rationale development of much needed drugs for this serious disease.

As described earlier, other infections due to *Acanthamoeba* are granulomatous amoebic encephalitis (GAE) that almost always proves fatal. However, there are no recommended treatments for GAE. This is due to low sensitivity of *Acanthamoeba* to many antiamoebic agents and the low level of ability of these agents to cross the blood-brain barrier into the central nervous system. Recent studies have indicated that alkylphosphocholine compounds, such as hexadecylphosphocholine, cross the blood-brain barrier and have *in vitro* antiamoebic activity (Walochnik *et al.,* 2002; Kotting *et al.,* 1992). Further studies are needed to determine their precise mode of action on *Acanthamoeba*, methods of application

and more importantly, success of these agents *in vivo* studies. Overall these studies have indicated the obvious need to find alternative means for successful treatment of these potentially devastating infections.

6. Ubiquity

As described in the introduction, *Acanthamoeba*e are found in diverse environments, from drinking water to distilled water wash bottles. It is not surprising that we often come across and interact with these organisms. Previously Chappell *et al.,* (2001), showed that > 80 % of the normal human population exhibited antibodies against *Acanthamoeba*. This clearly indicated that these are one of the most ubiquitous organisms and that they often come in contact with humans.

7. Biofilm formation

Biofilms are known to play an important role in the pathogenesis of *Acanthamoeba* keratitis. Biofilms are microbially derived sessile communities, which can be formed in aqueous environments as well as on any materials and medical devices including intravenous catheters, contact lenses, scleral buckles, suture material, and intraocular lenses (Zegans *et al.,* 2002). With reference to contact lenses, biofilms are formed through contamination of the storage case. Once established, biofilms provide attractive niches to *Acanthamoeba*, by fulfilling their nutritional requirements as well as providing resistance to disinfectants. In support, Beattie *et al.,* (2003) have recently shown that *Acanthamoeba* exhibited significant higher binding to worn and *Pseudomonas* biofilm-coated hydrogel lenses as compared to unworn lenses clearly indicating that biofilms enhances *Acanthamoeba* adherence to contact lenses. In addition, abundance of nutrition provides means for the transformation of *Acanthamoeba* into the vegetative, infective trophozoite form. It is important to indicate that *Acanthamoeba* adherence to human corneal epithelial cells most likely occurs during the trophozoite stage as cysts exhibit no and/or minimal binding (unpublished data). Overall, these findings suggest that biofilms play an important role in *Acanthamoeba* keratitis in contact lens users and intervening this process will provide preventive strategies.

8. Host factors

As described earlier, the ability of *Acanthamoeba* to cause infection is equally dependent on the host factors as it is on the parasite. However, the extent to which host factors contribute to the outcome of *Acanthamoeba* infections is unclear. This is due to the fact that host factors are more complex and difficult to study than those of the parasite. For example, in *Salmonella* infections, the genetic constitution of the host determines susceptibility to infection (Harrington and Hormaeche, 1986). However, these studies were possible only due to the availability of transgenic animals. There are multiple host factors, which may contribute to *Acanthamoeba* infections. For example, >90% of *Acanthamoeba* keratitis is known to occur in people who wear contact lens. In comparison, granulomatous amebic encephalitis due to *Acanthamoeba* has been reported only in immunocompromised individuals. In addition, malnutrition, mental stress, age, metabolic factors, and other primary infections may play a role in the pathogenesis of *Acanthamoeba* infections.

CONCLUSIONS

Acanthamoeba pathogenicity is a sum of multiple processes, which must come together in time and space for the

successful transmission of the pathogens to a susceptible host, overcome host barriers and cause disease. One of the key factors in *Acanthamoeba* pathogenesis is its ability to adapt to diverse conditions, both under stress, by transformation into cysts and emerging as vegetative trophozoite form under favourable conditions. We are only beginning to appreciate the complex nature of these organisms and the associated mechanisms that are employed to produce human diseases. This has been made possible due to recent developments in the availability of both *in vitro* and *in vivo* models of *Acanthamoeba* keratitis. In particular, the use of corneal epithelial cells *in vitro* assays has and will continue to provide insights into the precise mechanisms of *Acanthamoeba* pathogenesis, which may provide tools for the successful development of therapeutic interventions.

Studies to date have identified that binding of *Acanthamoeba* to host cells is mediated by mannose-binding protein that leads to secondary events such as phagocytosis, apoptosis and secretion of toxins such as extracellular proteases, which play a more direct role in host cellular and tissue damage. Extracellular proteases are considered both as necrotic as well as apoptotic agents and are major determinants in the pathogenesis of *Acanthamoeba* infections. However, *Acanthamoeba* are opportunistic organisms and it is likely that these enzymes may have evolved for nutritional purposes i.e., degradation of food particles and thus their major role in infection is host tissue degradation. Further investigations require understanding of their basic biochemical mechanisms, their relevance to each other, ability to cause disease and their interactions with host defense systems.

In addition, future studies are needed to identify the genetic basis for these virulence factors to produce disease. To show the importance of these virulence factors, it is crucial to show simultaneous loss of the factor and loss of virulence and complementing this property with regain of the factor. These will require precise genetic manipulations of single or multiple virulence genes, which will further assess the contribution of these virulence factors in the pathogenesis of *Acanthamoeba* infections. Since both parasite and host factors seems to be equally important in the pathogenesis of *Acanthamoeba* infections, it is reasonable to predict that emerging genomics will have the potential to provide insights into these vision-threatening and fatal infections. Further research in such areas will undoubtedly provide strategies to design novel therapeutic interventions.

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