

The immune response to *Naegleria fowleri* amebae and pathogenesis of infection

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Introduction

The genus *Naegleria* consists of a group of free-living ameboflagellates (FLA) found in diverse habitats throughout the world. *Naegleria* spp. have been isolated from freshwater lakes, ponds, domestic water supplies, swimming pools, thermal pools, soil, and dust (Marciano-Cabral, 1988; Rodriguez-Zaragoza, 1994; Szenasi *et al.*, 1998). Although over 30 species of *Naegleria* have been isolated from environmental sources, only *Naegleria fowleri* has been isolated from humans. *Naegleria fowleri* causes primary amebic meningoencephalitis (PAM), a rapidly fatal disease of the central nervous system (CNS) that occurs more often in children and young adults with a history of swimming and diving in freshwater. Two other species of *Naegleria*, *Naegleria australiensis* and *Naegleria italica*, can cause infection in experimental animals but have not been associated with disease in humans (De Jonckheere, 2004). There are three morphological stages in the life cycle of *Naegleria* – a trophozoite, a flagellate, and a cyst (Martinez, 1985) (Fig. 1). The trophozoite is the feeding, dividing, and presumably infective stage for humans. However, it is conceivable that

Abstract

The genus *Naegleria* is comprised of a group of free-living ameboflagellates found in diverse habitats worldwide. Over 30 species have been isolated from soil and water but only *Naegleria fowleri* (*N. fowleri*) has been associated with human disease. *Naegleria fowleri* causes primary amebic meningoencephalitis (PAM), a fatal disease of the central nervous system. The pathogenesis of PAM and the role of host immunity to *N. fowleri* are poorly understood. Strategies for combating infection are limited because disease progression is rapid and *N. fowleri* has developed strategies to evade the immune system. The medical significance of these free-living ameboflagellates should not be underestimated, not only because they are agents of human disease, but also because they can serve as reservoirs of pathogenic bacteria.

cysts also can enter the nares, convert to trophozoites and proceed to invade the brain. Surface structures on trophozoites, termed ‘food-cups’ (Fig. 2), are used to ingest bacteria and yeast in the environment as well as tissue in the infected host (Marciano-Cabral, 1988). Trophozoites characteristically undergo transformation from amebae to flagellates under conditions of nutrient deprivation in water and encyst under adverse environmental conditions (Dingle & Fulton, 1966). Human disease caused by *N. fowleri* was first reported by Fowler & Carter (1965) in South Australia. Subsequently, cases were reported in the United States in Florida (Butt, 1966), Texas (Patras & Andujar, 1966) and Virginia (Callicott *et al.*, 1968). Butt (1966) termed the disease PAM to distinguish this primary infection of the CNS from that resulting from secondary invasion of the brain by the intestinal parasite, *Entamoeba histolytica*. As a waterborne disease, the majority of cases of PAM have occurred in association with swimming and diving in inadequately chlorinated pools, contaminated spas and canals, or from exposure to environmental water sources during recreational activities such as water skiing (Cerva & Novak, 1968; Lares-Villa *et al.*, 1993; Gyori, 2003; Yoder

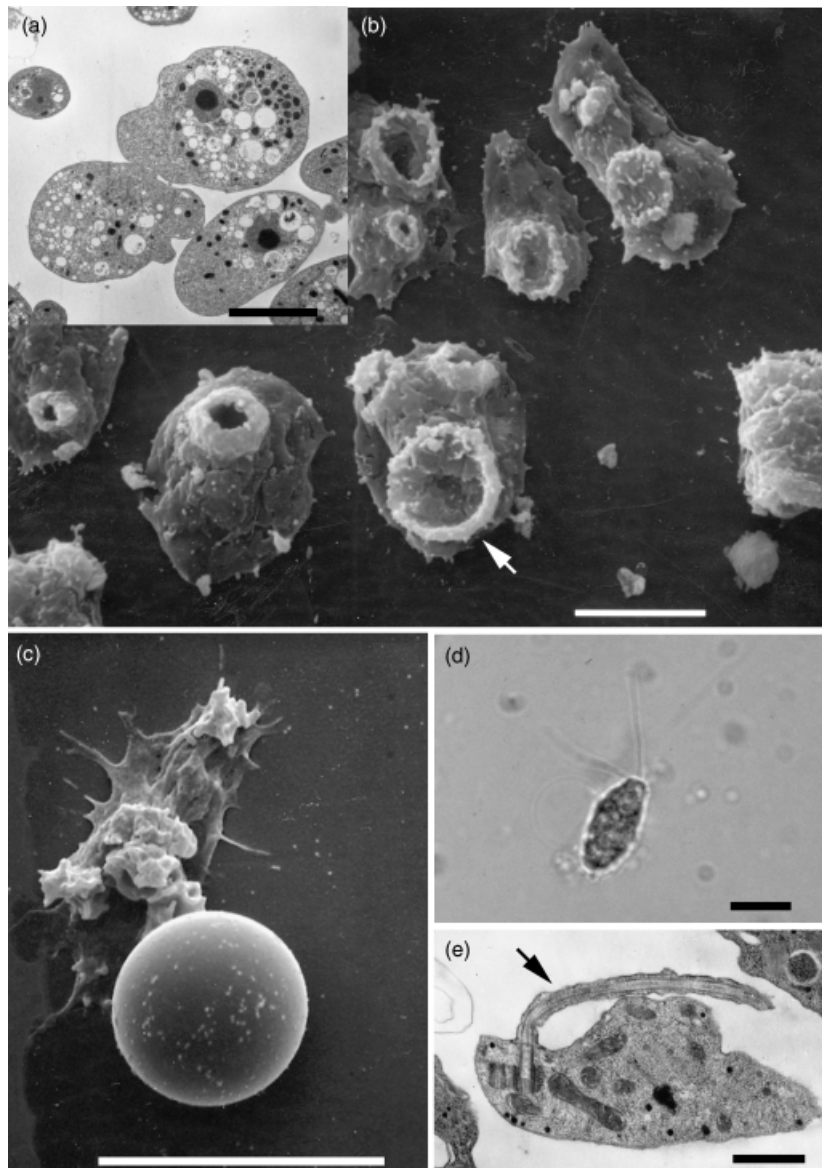


Fig. 1. Depiction of *Naegleria fowleri* in its three states of transformation. (a) Transmission electron micrograph (TEM) of trophozoites illustrating the prominent nucleus with a centrally located electron-dense nucleolus. (b) Scanning electron micrograph (SEM) of trophozoites exhibiting 'food-cups' (arrow). (c) SEM of a cyst. (d) Light micrograph of a flagellate with the characteristic two flagella. (e) TEM of flagellate illustrating one of the flagella (arrow). The scale bars represent 10 μm for (a–d) and 2 μm for (e).

et al., 2004; Craun *et al.*, 2005). Cerva & Novak (1968) reported 16 cases of PAM for which infection was traced to the same swimming pool in the Czech Republic. Cases of PAM, have also been linked to domestic water supplies (Anderson & Jamieson, 1972; Marciano-Cabral *et al.*, 2003).

Naegleria fowleri has been termed an amphizoic ameba because it can exist in a free-living state in water or soil or as a pathogen in human or animal hosts (Page, 1974). Other FLA found in the environment such as *Balamuthia mandrillaris*, *Sappinia diploidea*, and several species of *Acanthamoeba* are also capable of producing CNS infections in humans.

Balamuthia and *Acanthamoeba* are causative of the fatal granulomatous amebic encephalitis (GAE) (Marciano-Cabral & Cabral, 2003; Visvesvara *et al.*, 2007) while *Sappinia diploidea* has been identified as causative of a nonfatal infection of the CNS (Gelman *et al.*, 2003). While GAE occurs more frequently in immune-suppressed patients, PAM occurs in immune-competent individuals and elicits a more rapid disease course. Infection with *Naegleria* is initiated by the introduction of water containing organisms into the nasal cavity of the host. Amebae attach to the nasal mucosa, migrate along the olfactory nerves, cross the

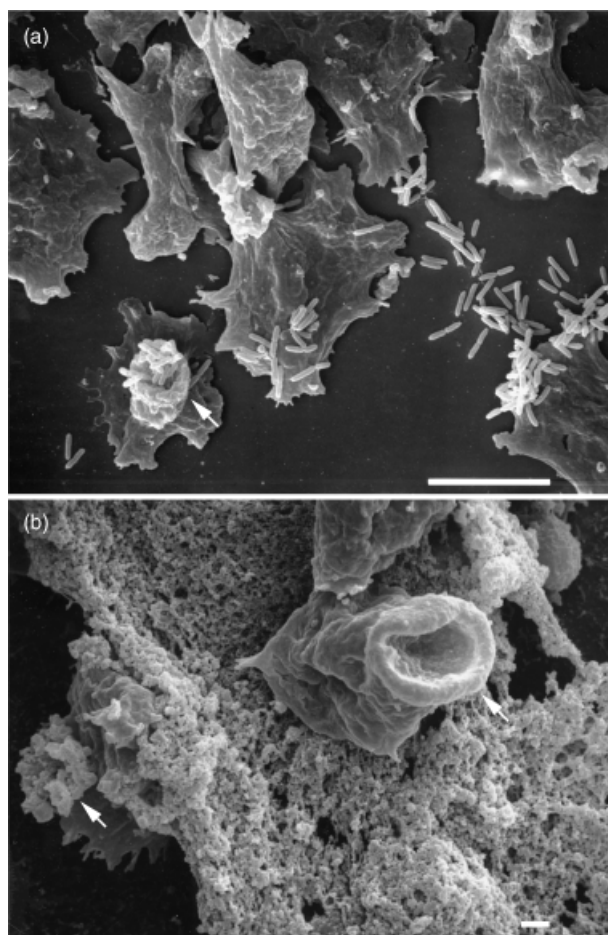


Fig. 2. Scanning electron micrographs of 'feeding' *Naegleria fowleri* trophozoites. (a) Trophozoite in the apparent process of ingesting *Escherichia coli* using a 'food-cup' (arrow). (b) Trophozoite with a prominent 'food-cup' observed on a brain fragment that was isolated from a moribund mouse 5 days postintranasal instillation with *N. fowleri*. Trophozoites (arrows) are attached to lysed brain tissue. The scale bars represent 10 µm for (a) and 1 µm for (b).

cribriform plate, and enter the brain. Only trophozoites are observed in the brain by hematoxylin and eosin staining of tissue. Once in that compartment, the amoebae cause extensive tissue damage and inflammation. Trophozoites lyse and ingest erythrocytes and other cell types such as nerve cells. The destruction of tissue and hemorrhagic necrosis of the brain is accompanied by an inflammatory infiltrate that consists of neutrophils, eosinophils, and macrophages. PAM is characterized by severe frontal headache, fever, nausea and vomiting, stiff neck, and occasional seizures. The acute hemorrhagic necrotizing meningoencephalitis that follows invasion of the CNS generally results in death 7–10 days postinfection (Carter, 1970; Martinez, 1985; Barnett *et al.*, 1996; Martinez & Visvesvara, 1997).

PAM in humans is difficult to identify because disease progression is rapid and the diagnosis is generally made post

mortem following hematoxylin and eosin staining of brain tissue. Martinez & Visvesvara (1997) recommended that direct microscopic examination be performed on wet-mount preparations of cerebrospinal fluid. Using this approach, a diagnosis of PAM could be made on identification of motile trophozoites, followed by specific immunofluorescent staining. In addition, a commercially available enzyme-linked immunosorbent assay based on the use of a monoclonal antibody (5D12) that recognizes a repeated glycosylated epitope present on proteins of *N. fowleri* can be used to diagnose infections (Sparagano *et al.*, 1993; Reveiller *et al.*, 2003). More recently, molecular techniques such as PCR and real-time PCR have been developed for the detection of *N. fowleri* in environmental and clinical samples (Pelandakis & Pernin, 2002; Reveiller *et al.*, 2002; Behets *et al.*, 2006). Pelandakis *et al.* (2000) designed a PCR assay using ribosomal internal transcribed spacers of *N. fowleri*. A PCR assay that detects *N. fowleri* in fresh brain tissue as well as in formalin-fixed paraffin-embedded brain tissue has also been developed (Schild *et al.*, 2007). In summary, *N. fowleri* CNS disease progression is rapid, and the availability of rapid diagnostic techniques affords an opportunity to apply prompt treatment that is essential for a successful patient outcome. There have been few survivors of PAM but survival has occurred when the disease was recognized early and treatment instituted promptly (Schuster & Visvesvara, 2004). To date, the drug of choice for treatment of PAM has been Amphotericin B in combination with rifampin and other antifungal agents (Seidel *et al.*, 1982). Intravenous injection of Amphotericin B and fluconazole, followed by oral administration of rifampin resulted in successful treatment of a 10-year-old child who developed PAM (Vargas-Zepeda *et al.*, 2005).

Several animal models of PAM have been developed. These include mice (Martinez *et al.*, 1971), monkeys (Culbertson, 1971), guinea pigs (Diffley *et al.*, 1976), sheep (Simpson *et al.*, 1982) and rabbits (Smego & Durack, 1984). While all of these experimental animals have proven susceptible to infection, the mouse model has been used most extensively due to ease of handling and development of a fatal disease that resembles PAM in humans following intranasal instillation of *N. fowleri* (Martinez *et al.*, 1971, 1973) (Fig. 3). Thus, studies using mice have yielded the largest body of data on the pathogenicity of environmental *Naegleria* isolates, disease progression, and host susceptibility or resistance to infection during drug treatment or immunization (Martinez, 1985).

Pathogenicity

The pathogenesis of PAM is poorly understood. Both pathogenic and nonpathogenic species of *Naegleria* have been isolated from the environment, but the determinants

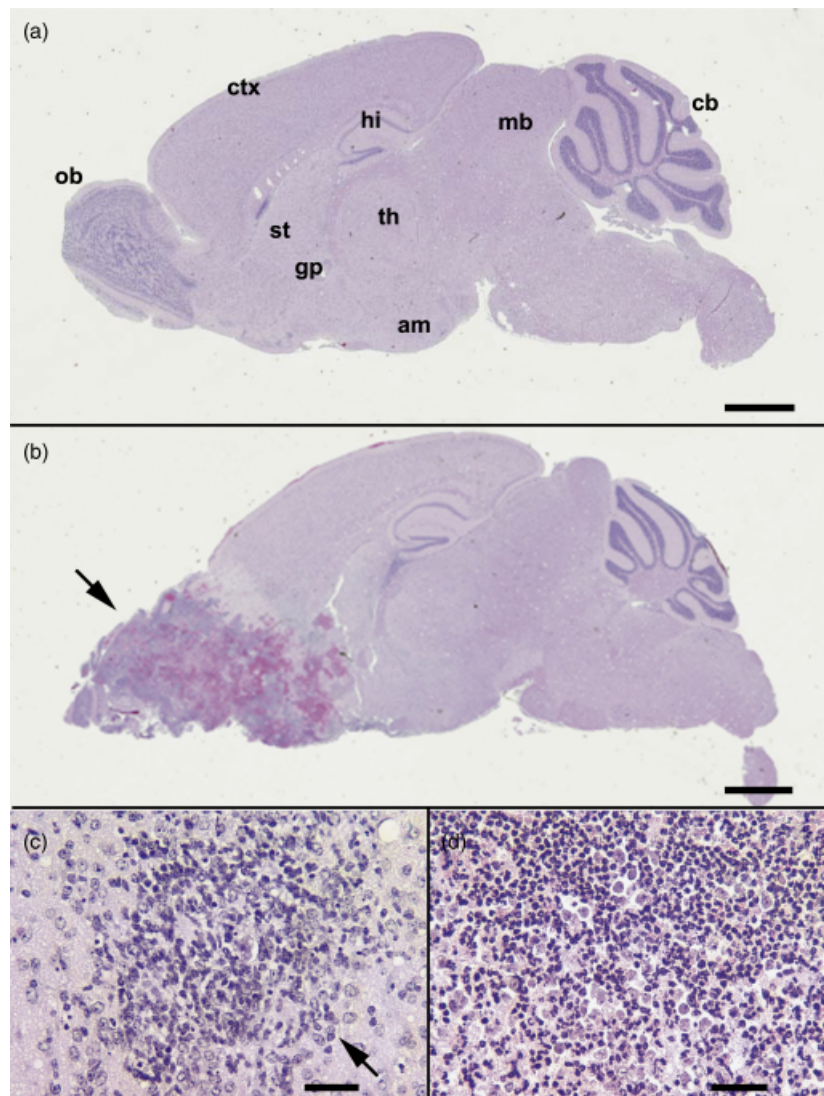


Fig. 3. Light microscopy histopathology of (B₆C₃)F₁ mice inoculated intranasally with *Naegleria fowleri*. (a, b) Whole-brain sagittal sections. (a) Uninfected mouse. (b) Infected mouse at 10 days postnasal instillation with trophozoites. Note the massive neuropathology (arrow) in the olfactory area and frontal lobe of the brain. (c) A focal lesion (arrow) in the striatum (st) of a mouse at 4 days postintranasal instillation with trophozoites. (d) Extensive accumulation of mononuclear and polymorphonuclear cells in striatum of mouse at 7 days postintranasal instillation with trophozoites. Note the numerous trophozoites admixed with the mononuclear and polymorphonuclear cells. All sections were stained with hematoxylin and eosin. The bars designate 2 mm for (a) and (b) and 10 μm for (c) and (d). am, amygdala; cb, cerebellum; ctx, cerebral cortex; gp, globus pallidus; hi, hippocampus; ob, olfactory bulb; th, thalamus; st, striatum.

of virulence and pathogenicity are unknown. As with many pathogenic organisms, prolonged growth of *N. fowleri* in axenic culture *in vitro* results in attenuation of virulence while serial passage through mice restores and maintains virulence (Wong *et al.*, 1977; Whiteman & Marciano-Cabral, 1987). Song *et al.* (2007) identified heat-shock protein 70 (HSP70) in *N. fowleri* and suggested that it played an essential role in adaptive responses for survival of amoebae, including protein folding and regulation of the host's immune system. However, temperature tolerance does not

appear to be a factor in pathogenicity because *Naegleria lovaniensis*, a species that shares thermophilic (i.e. grows at temperatures of 40 °C or higher) and antigenic properties with *N. fowleri*, is nonpathogenic in experimental animals (Stevens *et al.*, 1980).

Adherence of pathogens to host cells is a critical initial step in the infection process. The ability of trophozoites to attach to the nasal mucosa, an increased rate of locomotion, and a chemotactic response to nerve cell components may be important in disease progression (Cline *et al.*, 1986;

Brinkley & Marciano-Cabral, 1992; Han *et al.*, 2004). During tissue invasion, trophozoites apparently gain access to extracellular matrix glycoproteins (ECM) such as fibronectin, collagen, and laminin, which are found at the basal lamina and surrounding cells in tissue. Han *et al.* (2004) used binding assays to investigate the interaction of *N. fowleri* with fibronectin *in vitro* and found that the number of amoebae bound to fibronectin was increased compared with controls. In addition, a fibronectin-binding protein of 60 kDa was identified in extracts of *N. fowleri*. Finally, the investigators obtained strong inhibition of amoebic binding to fibronectin using an antibody to integrin α_5 , indicating that *N. fowleri* possesses a surface protein that is similar to the human integrin-like receptor that mediates adhesion to fibronectin.

A variety of *in vitro* cell culture systems have been used to study the interaction of *N. fowleri* with mammalian cells. *Naegleria fowleri* trophozoites have been shown to destroy nerve cells, as well as other cell types, by trophocytosis (i.e. piecemeal ingestion) using a 'food-cup' structure on their surface (Brown, 1979; Marciano-Cabral *et al.*, 1982; Marciano-Cabral & Fulford, 1986) and by the release of cytolytic molecules (Lowrey & McLaughlin, 1984, 1985; Fulford & Marciano-Cabral, 1986; Leippe & Herbst, 2004). The mode that is applied to destroy target cells *in vitro*, however, is dependent on the amoeba strain. For example, weakly pathogenic strains destroy nerve cells by ingestion using the 'food-cup' structure while highly pathogenic strains lyse nerve cells on contact and subsequently ingest the cell debris that is generated (Marciano-Cabral *et al.*, 1982; Marciano-Cabral, 1988). A variety of proteins has been implicated in playing a role in amoeba-mediated lysis of target cells. A membrane-associated pore-forming protein from *N. fowleri* was reported to exhibit hemolytic activity for red blood cells (Ding-E Young & Lowrey, 1989). Herbst *et al.* (2002, 2004) isolated and characterized two pore-forming proteins, designated naegleriapores A and B, that were shown to be cytotoxic to human cells. In addition to naegleriapores, it has been reported that *N. fowleri* secrete proteases (Aldape *et al.*, 1994; Mat Amin, 2004), acid hydrolases (Feldman, 1977; Lowrey & McLaughlin, 1985), phospholipases (Chang, 1979; Hysmith & Franson, 1982a; Fulford & Marciano-Cabral, 1986; Barbour & Marciano-Cabral, 2001), and phospholipolytic enzymes that degrade sphingomyelin (Hysmith & Franson, 1982b). While these enzymes may be associated with pathogenicity, none appears to be unique to pathogenic species of *Naegleria* (Chang, 1979). However, Cursons *et al.* (1978) reported that pathogenic *N. fowleri* produce higher levels of phospholipase A and lysophospholipase as compared with nonpathogenic *Naegleria* spp. Eisen & Franson (1987) indicated that pathogenic *Naegleria* secreted neuraminidases, which, along with lipases, were considered as contributing to glycolipid and phospholipid

alterations associated with demyelinating disease. Ferrante & Bates (1988) detected elastase in *N. fowleri*, an enzyme that degrades a wide range of connective tissue proteins such as collagen and proteoglycans, and suggested that it could play a role in tissue destruction and invasion. Mat Amin (2004), using gelatin gels, reported the finding of two high-molecular-weight cysteine proteinases (128 and 170 kDa) and suggested that these proteinases could be involved in tissue destruction and pathogenesis. In addition, the presence of a 30 kDa cysteine protease in conditioned medium of *N. fowleri* has been reported (Aldape *et al.*, 1994). This protease was found to catalyze the degradation of extracellular matrix proteins *in vitro* and to produce a cytopathic effect on mammalian cells. In summary, a diverse array of protein entities has been implicated as virulence factors associated with the cytopathology of a variety of mammalian target cells. It is possible that naegleriapores, proteases, and phospholipases act in tandem to facilitate invasion of the host and cause cell destruction.

A number of gene expression approaches have been considered in attempts to discriminate between pathogenic and nonpathogenic *Naegleria*. Yun *et al.* (2002) examined differences in the gene expression of highly pathogenic mouse-passaged *N. fowleri*, weakly pathogenic *N. fowleri*, and nonpathogenic *Naegleria gruberi*. Northern blot hybridization analysis, performed on cDNA clones randomly selected from a cDNA library, was used to compare the expression patterns of *N. fowleri* genes isolated before and after passage through mouse brain. Genes that demonstrated constant levels of expression during mouse passage included a ribosomal protein, fumarase, malate dehydrogenase, and ubiquitin. Expression of two genes, high mobility group protein (HMG) and the 26S proteasome subunit, was observed to increase in pathogenic *N. fowleri* upon continuous mouse passage. The investigators suggested that the identification of expression of genes encoding the 26S proteasome subunit and ubiquitin was indicative of the fact that protein turnover occurs at a higher rate in pathogenic *N. fowleri* as compared with nonpathogenic *N. gruberi*. The HMG gene product may serve as a transcription factor, as has been found in other organisms, because two DNA-binding domains were well conserved in the *N. fowleri* protein. Nevertheless, although the levels of HMG and the 26S proteasome subunit were increased correlative to increased mouse brain passage, a linkage of these gene products to virulence could not be made (Yun *et al.*, 2002). The construction of cDNA expression libraries as an approach to identify genes expressed by pathogenic *N. fowleri* has also been reported. Shin *et al.* (2001) examined clones for *N. fowleri* proteins by immunoscreening. A cDNA clone thus identified and sequenced was designated nfa1 and was found to code for myohemerythrin. The authors suggested that, because myohemerythrin is an O₂⁻ carrying protein,

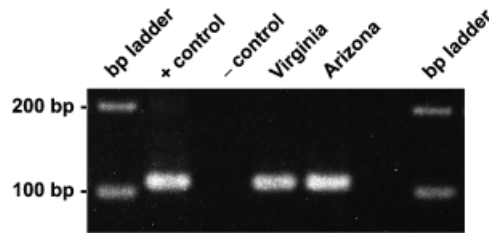


Fig. 4. Detection of *Naegleria fowleri* in water sources using PCR. Nested PCR was performed on an environmental water source (VA) and on a domestic water source (AZ) using *N. fowleri*-specific primers as described by Reveiller *et al.* (2002). The ethidium bromide-stained 1.5% agarose gel depicts the 110-bp amplification product indicative of *N. fowleri* DNA. DNA from clone Mp2c15 served as a positive (+) control while PCR-graded water lacking DNA served as the (-) control.

Nfa1 could play a role in the survival of the amoebae. Using a polyclonal antibody produced to recombinant Nfa1 protein, the protein was localized by immunohistochemistry to the pseudopodia and the area surrounding the food vacuoles of *N. fowleri* trophozoites. Cho *et al.* (2003) reported that *N. fowleri* cocultured with Chinese Hamster ovary (CHO) cells in the presence of an anti-Nfa1 polyclonal antibody exhibited reduced cytotoxic activity for the CHO target cells. Reveiller *et al.* (2001) obtained a unique sequence termed mp2c15 from a cDNA library that was constructed from a highly pathogenic mouse-passaged strain of *N. fowleri*. The sequence was found not to match any currently available in the BLAST database. Mp2c15 was found to code for a 17 kDa protein in *N. fowleri* but not in pathogenic or nonpathogenic species of *Acanthamoeba*, *Vahlkampfia*, *Vanella*, *Balamuthia*, or *Hartmanella* (Reveiller *et al.*, 2001; MacLean *et al.*, 2004). Using primers specific to the *N. fowleri* mp2c15 sequence, a PCR assay was developed that identifies *N. fowleri* in water and soil samples (Fig. 4) (Reveiller *et al.*, 2001). Nevertheless, although a number of gene products have been identified in *N. fowleri* using molecular cloning strategies, whether any of these plays a role in virulence or pathogenicity remains to be determined.

The virulence of *N. fowleri* may be associated with fluctuations in the levels of select proteins rather than an all-or-none differential expression of select genes. Hu *et al.* (1991, 1992) found that gene expression for a serine carboxypeptidase increased in highly virulent *N. fowleri* and when mammalian cells were used as a food source for amoebae in culture. Using two-dimensional polyacrylamide gel electrophoresis, changes were observed in protein profiles between highly virulent and weakly virulent *N. fowleri*. In particular, a loss of protein species was observed for the highly virulent strain of *Naegleria* (Hu *et al.*, 1991). It was suggested that the loss of proteins may be a critical requisite for conversion of *Naegleria* to a more virulent state.

Immune response to *Naegleria*

Innate immunity

In addition to the ability to attach to nasal mucosa, to exhibit an increased rate of locomotion, and to destroy target cells by trogocytosis and by release of cytolytic molecules, *N. fowleri* may have evolved as a successful pathogen because it has developed mechanisms to evade the host immune system. It has been shown that *Naegleria* are resistant to lysis by host cytolytic molecules such as tumor necrosis factor (TNF)- α , IL-1, and the membrane attack complex (MAC) C5b-C9 of complement (Fischer-Stenger & Marciano-Cabral, 1992; Toney & Marciano-Cabral, 1992). The body of currently available data suggests that innate immunity may play a greater role than acquired immunity in resistance to *N. fowleri* infection. Components of the innate immune system that have been shown to respond to infection with *N. fowleri* include complement, neutrophils, and macrophages.

Complement

The complement system, which constitutes a first line of defense against invading organisms, is activated by *Naegleria* trophozoites (Holbrook *et al.*, 1980; Rowan-Kelly *et al.*, 1980; Whiteman & Marciano-Cabral, 1987). Studies as early as 1978, using the mouse model of PAM, showed that the most susceptible mouse strain (A/HeCr) for *N. fowleri* infection was complement (C5) deficient (Haggerty & John, 1978). Furthermore, mice depleted of complement by cobra venom factor were found to be more susceptible to infection with *N. fowleri* than were noncomplement-depleted mice (Reilly *et al.*, 1983b). These *in vivo* observations are in accord with those obtained *in vitro*, which have indicated that nonpathogenic and weakly pathogenic species of *Naegleria* are complement-sensitive and are lysed by the MAC of complement (Whiteman & Marciano-Cabral, 1987). However, *N. fowleri* that have been passaged through experimental animals and thus are highly pathogenic, are more resistant to the lytic effect of complement (Whiteman & Marciano-Cabral, 1989). These observations are in agreement with those obtained for a number of microorganisms and eukaryotic cells that have developed mechanisms to evade complement lysis. Eukaryotic cells including mammalian erythrocytes, neutrophils, and tumor cells have been shown to possess complement-regulatory proteins that protect them from lysis. For example, CD59, an 18 kDa complement-regulatory protein found on the surface of human erythrocytes and leukocytes, has been reported to protect these cells from lysis by complement (Meri *et al.*, 1990). These regulatory proteins on the surface of cells reportedly interfere with complement-mediated lysis by

preventing activation of complement or preventing lysis once complement is activated (Kim & Song, 2006).

Studies *in vitro* have shown that highly pathogenic *Naegleria* amebae apply at least two means to resist complement damage: (1) expression of complement-regulatory proteins and (2) shedding of the MAC (C5b-C9) on vesicles (Toney & Marciano-Cabral, 1992, 1994). Toney & Marciano-Cabral (1992) reported that proteins on the surface of highly pathogenic *N. fowleri* played an important role in trophozoite resistance to complement lysis. The treatment of highly pathogenic *N. fowleri* with enzymes such as trypsin, papain, endoglycosidase H, or phosphatidylinositol-specific phospholipase c to remove surface components resulted in an increase in the susceptibility of complement-resistant amebae to complement-mediated lysis. Recently, Fritzinger *et al.* (2006) used a monoclonal antibody for human CD59 to identify an 18 kDa immunoreactive protein on the surface membrane of *N. fowleri*. The protein was designated a 'CD59-like protein' because it reacted with antibodies to human CD59. However, it is yet to be determined whether this protein is functionally relevant in resistance to complement-mediated lysis of amebae. While the role of human CD59 in complement regulation is well known, studies indicate that it also has signaling properties. Consistent with these observations, it has been reported that *N. fowleri* possesses serine/threonine and tyrosine protein kinases (Chu *et al.*, 2000). It was suggested that the 'CD59-like' molecule interacts with protein kinases, resulting in vesiculation and removal of the lytic complex, C5b-C9, from the ameba membrane surface. Chu *et al.* (2000) demonstrated that differential activation of signal transduction pathways occurred in amebae in response to serum complement. The increased activity of tyrosine kinases after treatment with serum complement was inhibited with kinase inhibitors and was accompanied by increased susceptibility of the amebae to complement lysis. Collectively, these results indicate that these kinases may play a critical role in the protection of *N. fowleri* ameba to complement-mediated lysis. The 'CD59-like' protein is also expressed when *N. fowleri* is cocultured with bacteria that produce toxins (Fritzinger & Marciano-Cabral, 2004). Fritzinger *et al.* (2006) suggested that the 'CD59-like' protein could protect amebae, not only from the action of the membrane attack complex of complement (C5b-C9) but also from that of pore-forming proteins such as bacterial toxins and the pore-forming proteins produced by *Naegleria* amebae known as naegleriapores.

In addition to the presence of cell surface proteins that inhibit the complement pathway, a variety of regulatory mechanisms have been identified that protect cells from damage by the MAC of complement. The mechanisms that have been described include the ability of cells to shed the MAC from the cell surface in the form of membrane blebs or

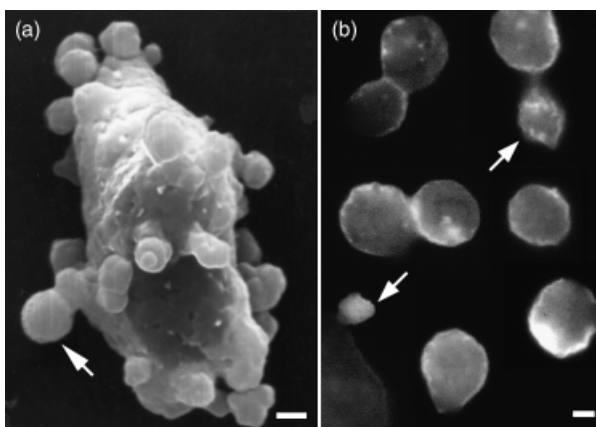


Fig. 5. Detection of the MAC of complement on the membrane of *Naegleria fowleri* amebae. Trophozoites were incubated with normal human serum diluted 1 : 4 for 60 min. (a) Scanning electron micrograph illustrating numerous bleb structures (arrow) extending from the surface of a trophozoite. (b) Immunofluorescence micrograph of trophozoites stained with polyclonal rabbit antiserum (anti-MAC) to human MAC proteins (c5b-c9), followed by FITC-labeled goat anti-rabbit IgG antibody. Note the presence of an immunoreactive product on the surface of trophozoites as well as on bleb structures (arrows) extending from the trophozoite surface. The scale bars represent 2 μ m for (a) and (b).

vesicles, endocytosis and degradation of membrane-bound C5b-C9, and the inability of the MAC to become inserted into the cell membrane lipid bilayer due to steric hindrance (Morgan, 1989; Gasque, 2004). Toney & Marciano-Cabral (1994) demonstrated that *N. fowleri* amebae undergo a process of vesiculation as a means of removal of the lytic MAC of complement. Incubation of *N. fowleri* amebae in human serum complement resulted in vesicle formation on their surface (Fig. 5). Immunofluorescence analysis using an antibody to the membrane attack complex of complement (anti-C5b-C9) indicated that the vesicles contained the C5b-C9 complex. Thus, it appears that removal of the lytic complex protects the ameba from complement-mediated lysis.

Neutrophils

Histopathological examination of human brain lesions containing amebic trophozoites reveals an inflammatory infiltrate that consists of neutrophils, eosinophils, and macrophages. The activated neutrophils within these lesions may play an important role in the early phase of *Naegleria* infections. Martinez (1985) reported a marked increase in neutrophils in white blood cell counts during *Naegleria* infection and that neutrophils predominated in cerebrospinal fluid taken from infected individuals. These observations from human infection have been replicated for experimental infections in mice where neutrophils are abundant in tissue early in infection (Fig. 6a and b). A role

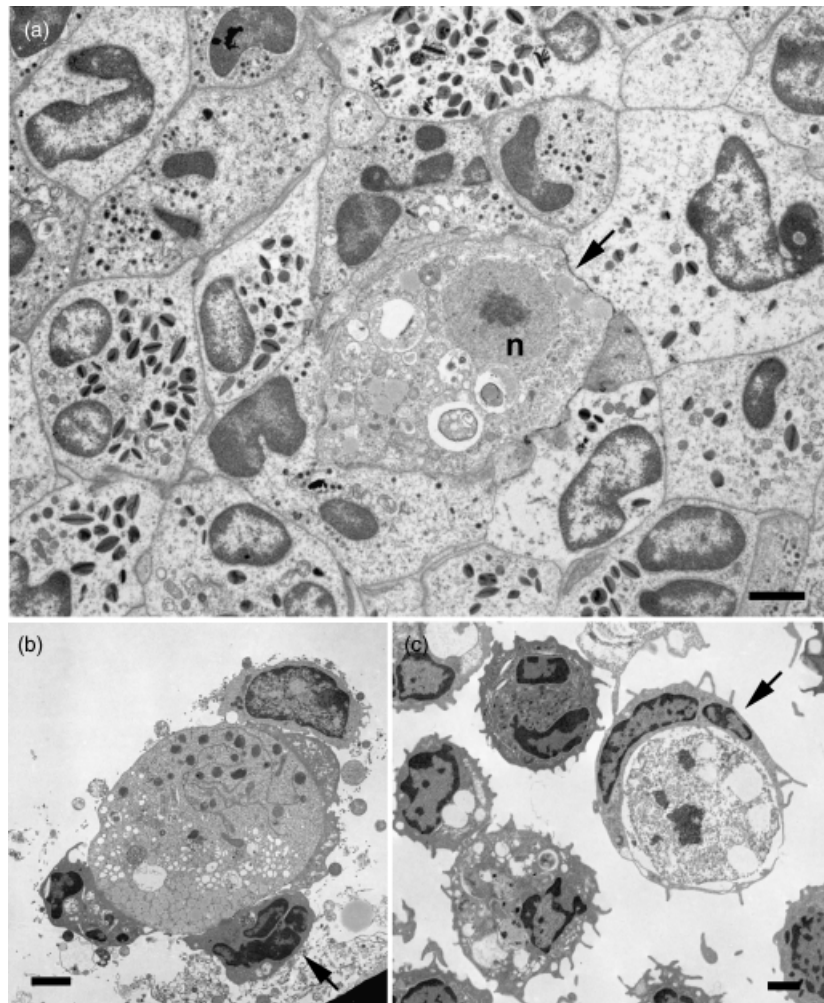


Fig. 6. Transmission electron micrographs depicting immune cell response to *Naegleria fowleri*. (a) Eosinophils, macrophages, and polymorphonuclear cells surround *N. fowleri* trophozoites (arrow). (b) *Naegleria fowleri* trophozoite attached to a polymorphonuclear cell that has the characteristics of a neutrophil (arrow). (c) A macrophage (arrow) in the apparent process of ingesting and digesting a trophozoite. The scale bars represent 2 μm . n, nucleus.

for neutrophils in immunity against *Naegleria* was reported by Ferrante & Mocatta (1984), who indicated that lymphokine-activated neutrophils were capable of killing *Naegleria in vitro*. Ferrante *et al.* (1987) further suggested that the myeloperoxidase– H_2O_2 –halide system of the neutrophil plays a major role in the expression of anti-amebic activity. $\text{TNF-}\alpha$ augmented the neutrophil response to *N. fowleri* (Ferrante, 1989). Michelson *et al.* (1990) reported that neutrophils activated *in vitro* with $\text{TNF-}\alpha$ adhered to *N. fowleri* and subsequently destroyed the amebae. However, while $\text{TNF-}\alpha$ appears not to have a direct effect on *N. fowleri*, destruction of amebae by neutrophils occurs only in the presence of this proinflammatory cytokine. Ferrante *et al.* (1988) reported that mice depleted of circulating

neutrophils, or abrogated of neutrophil function by treatment with a monoclonal antibody to neutrophils (NIMP-R10), experienced extensive amebic invasion of the brain and increased mortality upon challenge with *N. fowleri*. It has also been suggested that complement activation products elicit a strong chemotactic response resulting in the accumulation of neutrophils around amebae that results in their destruction (Rowan-Kelly *et al.*, 1980). Thus, while the definitive mechanism by which neutrophils exert anti-*Naegleria* activities remains to be defined, it is apparent that they play a role in the destruction of amebae and that the process of their aggregation at focal sites harboring amebae is mediated by a plethora of soluble factors including cytokines and complement components.

Macrophages

There is accumulating evidence that macrophages also act in host defense against *Naegleria*. There is a large body of data that indicates that activated macrophages serve as efficient cytotoxic cells against tumor cells and pathogens by producing reactive oxygen intermediates during the respiratory burst and eliciting nitric oxide (NO) and nonoxidative mediators including TNF- α and IL-1. Thus, it is not unexpected that activated macrophages also serve as important effector cells against *Naegleria* (Cleary & Marciano-Cabral, 1986a). Macrophages activated by different immunomodulators including *Mycobacterium bovis* bacillus Calm  tte–Gu  rin (BCG) or fixed *Propionibacterium acnes* bacteria destroy *Naegleria fowleri* amebae (Fig. 6c) by both a contact-dependent process and by secretion of soluble cytolytic factors (Cleary & Marciano-Cabral, 1986a, b). The amebicidal activity of macrophages appears to be due in part to an L-arginine-dependent pathway because the arginine analog N^GMMA, which acts as a selective inhibitor of L-arginine-dependent nitrogen oxidation, inhibits macrophage cytolytic activity against the amebae (Fischer-Stenger & Marciano-Cabral, 1992). Conditioned medium generated from activated macrophages upon treatment with bacterial lipopolysaccharide contains cytolytic factors that are amebicidal. Studies in which different macrophage populations have been used indicate that amebicidal factors produced by BCG-activated and *P. acnes*-activated macrophages may be distinct. Fischer-Stenger *et al.* (1992) reported that antiserum to TNF- α decreased the amebicidal activity of conditioned medium from BCG-activated macrophages stimulated with lipopolysaccharide but had no effect on conditioned medium from *P. acnes*-activated macrophages. Furthermore, addition of protease inhibitors resulted in a decrease in amebicidal activity in *P. acnes*-activated macrophage conditioned medium but had no effect on BCG-activated macrophage conditioned medium. The putative macrophage lytic molecule TNF- α , which lyses tumor cells, is apparently not amebicidal because recombinant TNF- α alone, or in combination with IL-1 α or IL- β , was neither cytolytic nor cytostatic for *N. fowleri*. Fischer-Stenger *et al.* (1992) suggested that BCG-activated, as well as *P. acnes*-activated, macrophages use a combination of nitric oxide and other cytolytic molecules to lyse amebae.

Studies using microglia, the resident macrophages of the brain, suggest that these cells may also play an important role in host resistance to *Naegleria* and, indeed, may comprise a first line of defense against CNS invasion. *In vitro* studies with primary neonatal rat microglia indicate that they can destroy weakly pathogenic *N. fowleri* amebae, whereas highly pathogenic, mouse-passaged *N. fowleri*, rather than serving as targets, destroy microglia. Highly pathogenic mouse-passaged *N. fowleri* also destroy brain

tissue as evidenced by electron microscopy (Fig. 2b). Multiprobe RNase protection assay (RPA) performed on total RNA obtained from cocultures revealed that both weakly pathogenic and highly pathogenic *N. fowleri* induced the production of proinflammatory cytokine mRNAs from microglia. Robust levels of message for the proinflammatory cytokines, IL-1 α , IL-1 β , IL-6, and TNF- α were produced. The production of message for proinflammatory cytokines was shown to be higher when highly pathogenic strains vs. weakly pathogenic strains were cocultured with microglia (Fig. 7) (Marciano-Cabral *et al.*, 2001). These *in vitro* results have been replicated using total RNA from whole-cell homogenates of murine brain. The production of these cytokines in the brain did not appear to render protection against infection. Oh *et al.* (2005) also reported that, although *N. fowleri* induced the production of TNF- α , IL-1 β , and IL-6, it nevertheless destroyed microglia by a phagocytic process. Addition of an antibody to the nfa1 gene product, myohemerythrin, to cocultures of amebae and microglia resulted in a reduction of the cytotoxic effect of *N. fowleri* amebae for microglia. Anti-Nfa1 also slightly decreased the secretion of TNF- α from microglia in response to the amebae. Oh *et al.* (2005) suggested that the Nfa1 protein (myohemerythrin) served as a critical target for a protective immune response of the host infected with *N. fowleri*. Collectively, these observations suggest that the production of proinflammatory cytokines in the brain in response to *N. fowleri* may not be protective. Indeed, the robust levels of these inflammatory factors may contribute to the disease process. Thus, *Naegleria* may first cause direct physical damage to surrounding neuronal cells and other cell types in the brain through direct effector cell–target cell contact or through the mediation of released soluble cytolytic factors. Second, *Naegleria* may elicit an immunopathological process in the brain. That is, these released cytotoxic factors, in concert with debris generated through lysis of neuronal cells and other cells in the brain, may serve to attract microglia to focal sites of infection. Consequent activation of microglia may lead secondarily to activation of astrocytes and the generation of a cascade of inducible expression of inflammatory cytokines that results in hyperinflammation, breakdown of the blood–brain barrier, and an influx of immune cells from nonneuronal sites.

On the other hand, it is not clear whether small numbers of amebae that are introduced into the brain cause subclinical infection, are destroyed by immune cells, or replicate to elicit a fatal infection. Dempe *et al.* (1982) demonstrated that mice inoculated intranasally with amebae of low virulence generated through maintenance in axenic culture for over 10 years survived infection. These mice, when tested in a labyrinth experiment, reportedly demonstrated a diminution in performance. Furthermore, amebae could be reisolated from the brains of these apparently ‘healthy’

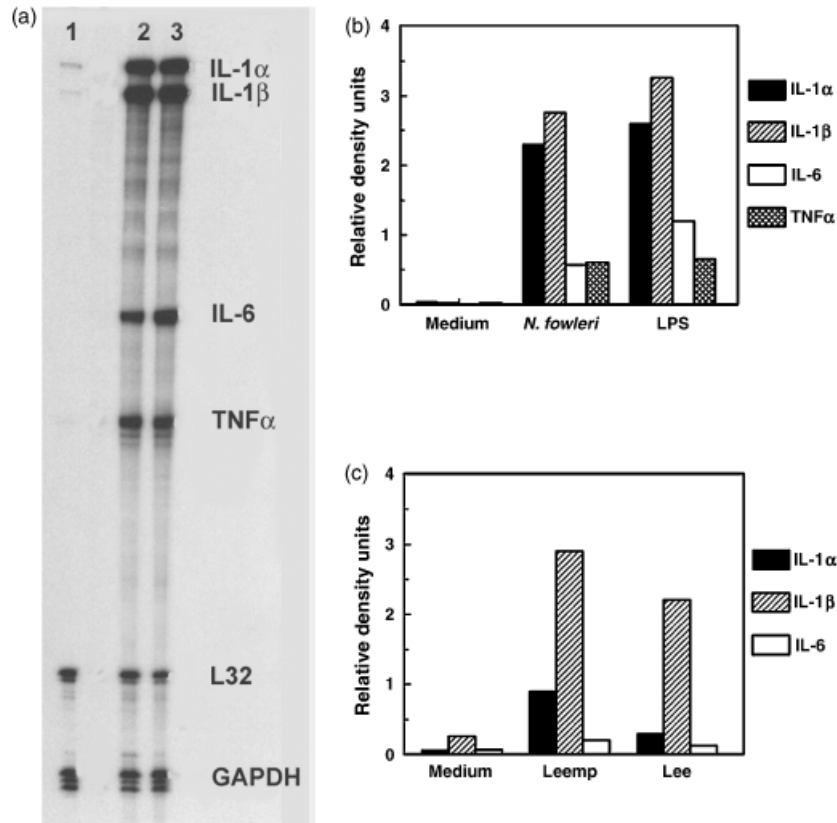


Fig. 7. Relative levels of cytokine mRNA elicited in response to *Naegleria fowleri*. (a) Microglial cells were harvested from neonatal rat brain, incubated at 37 °C in medium alone (lane 1) or in the presence of *N. fowleri* (Leemp) (lane 2) or bacterial lipopolysaccharide (10 ng mL⁻¹) (lane 3) for 6 h, and assessed for levels of proinflammatory cytokine mRNA by multiprobe RPA. (b) Graphic depiction of RPA results comparing microglial cytokine mRNA levels elicited by microglia alone or cultured in the presence of *N. fowleri* Leemp (i.e. mouse-passaged and highly pathogenic) or *N. fowleri* Lee (i.e. axenically grown and weakly pathogenic). While both weakly pathogenic and highly pathogenic *N. fowleri* induced mRNA for the proinflammatory cytokines IL-1α, IL-1β, TNF-α, and IL-6, the levels were higher for the highly pathogenic amoebae.

animals. The histopathological findings in the brain resembled features of chronic granulomatous amoebic encephalitis. Based on these observations, the authors reported that *N. fowleri* could cause latent infections. Thus, in animal models, it is apparent that strain differences in virulence occur and that low-virulence strains of *N. fowleri* may not cause lethal infections. However, in humans, it has not been established whether asymptomatic or chronic CNS infections occur. Nevertheless, it is likely that a respiratory or nonclinical infection can occur because amoebae have been isolated from the nasal passages of apparently healthy children (Chang *et al.*, 1975; Visvesvara & Healy, 1975; Lawande *et al.*, 1979).

Humoral immunity

The humoral immune response to *Naegleria* has been studied in humans and experimental animals. Serum samples from healthy individuals from the United States (Reilly *et al.*, 1983a; Marciano-Cabral *et al.*, 1987), New Zealand

(Cursons *et al.*, 1977, 1980a), and the Czech Republic (Cerva, 1989) have been examined for antibodies to *N. fowleri*. Although the antibody titers recorded have differed from study to study, almost all human sera from healthy individuals have been found to be positive for *N. fowleri*, indicating that exposure to the amoeba is common. *Naegleria* spp. have been identified as a source of 'humidifier fever,' a nonlethal hypersensitivity reaction to inhaled antigenic material in recirculating water in humidifier systems (Edwards *et al.*, 1976). Antigenic material from *Naegleria* has been shown to react, by gel diffusion assays, with sera from individuals exhibiting 'humidifier fever.' In another study of workplace-related 'humidifier fever,' positive serological tests did not correlate with symptoms but amoebae were found in all humidifiers studied (Finnegan *et al.*, 1987). Positive tests for amoebae correlated with a precipitin reaction to humidifier contaminants. However, it was suggested that the presence of multiple organisms, including *N. fowleri*, accounted for outbreaks of humidifier fever. Antibodies to *N. fowleri* have also been detected in 101 of 115

randomly obtained serum samples from hospitalized patients in the United States (Dubray *et al.*, 1987). Using immunoblot analysis, Powell *et al.* (1994) examined serum samples from army recruits with acute respiratory disease for antibodies to free-living amoebae. Antibodies to six different species of free-living amoebae, including *N. fowleri*, were detected.

A number of studies have been conducted to determine the role of antibody by idiotype in host resistance to infection with *Naegleria*. Rivera *et al.* (2001) conducted a survey to detect IgA antibodies in the serum and saliva of individuals living in Mexico in areas where *Naegleria* is endemic as compared with those where it is nonendemic. In amoebic endemic areas, titers of IgA antibody to *N. fowleri* in the serum and saliva were found to be significantly higher in individuals with upper respiratory tract infections as compared with those in healthy individuals. The investigators indicated that IgA and IgM present in mucosal secretions could play an important role in preventing amoebic infection by blocking adhesion of trophozoites to mucosal epithelium. Cursons *et al.* (1979) reported that serum obtained before death from a patient with PAM, which showed no increase in specific antibody titers by indirect immunofluorescent antibody assay, revealed very low levels of IgA upon radial-immunodiffusion testing for quantitation of serum IgM, IgG, and IgA. The investigators suggested that IgA deficiency could be a contributive factor to susceptibility to infection. In contrast, Cain *et al.* (1979) obtained serum before death from a patient with PAM and noted that the levels of serum immunoglobulins, including those of IgA, were within normal limits. Seidel *et al.* (1982), using immunofluorescence testing, reported an antibody titer of 1:4096 after 42 days of infection in a patient who survived PAM following treatment by intravenous and intraventricular administration of amphotericin B. Early recognition of the disease and expeditious application of treatment were considered to be important factors in the survival of this patient. In summary, although infection with *N. fowleri* results in elicitation of antibodies in humans, a correlation between susceptibility to PAM and humoral immune status or levels of secretory IgA has not been found.

In view of the limited data available regarding the role of humoral immunity in conferring resistance to *Naegleria* infection in humans, various investigators have availed themselves of animal models. The mouse model of PAM has been used to determine the effect of immunization with repeated administration of formalinized amoebae, freeze-thawed amoebic extracts, amoeba culture fluid, and sublethal doses of live amoebae (Thong *et al.*, 1980; Reilly *et al.*, 1983b). Although a marked humoral response was observed in mice, all immunization regimens produced only modest protection against challenge infection with *N. fowleri*. Reilly *et al.* (1983b) showed that the bulk of the elevated immunoglo-

bulins detected in mice stimulated with *Naegleria* antigens was not due to *Naegleria*-specific agglutinins but rather was the result of a nonspecific polyclonal B cell response. The investigators also showed that host resistance to infection with *N. fowleri* was not altered when mice were pretreated with cyclophosphamide or subjected to ^{60}Co radiation to inhibit the antibody response. Ferrante & Thong (1979) indicated that an antibody response does not appear to be a critical factor in host resistance because *N. fowleri* amoebae cap, internalize, and degrade antibody. Studies using a rabbit model of *N. fowleri*-induced meningitis to assess the efficacy of passive immune therapy administered intracisternally indicated that passive transfer of anti-*Naegleria* IgG or *Naegleria* monoclonal antibody prolonged the time to death but did not result in a cure for the infection (Lallinger *et al.*, 1987). The slight protective effect of antibody was attributed to immobilization of amoebae and prevention of their rapid migration to the brain, enhanced complement-mediated lysis, and neutralization of amoeba cytotoxins. In summary, the data derived from animal studies indicate that solid actively acquired immunity to *N. fowleri* has not been achieved even though immunized mice develop species-specific agglutinating antibodies. Collectively, the results from human and animal studies suggest that humoral immunity is not a major line of defense against *Naegleria*.

Cell-mediated immunity (CMI)

The role of CMI in responsiveness to *N. fowleri* has been studied *in vivo* using the delayed-type hypersensitivity response and *in vitro* using the macrophage migration assay. Diffley *et al.* (1976) demonstrated that guinea-pigs sensitized with intradermal injections of *N. fowleri* and other *Naegleria* species developed a delayed-type hypersensitivity skin reaction when challenged by an intradermal injection with homologous or heterologous *Naegleria* antigens. Cursons *et al.* (1980b) assessed the role of CMI using both an *in vitro* macrophage inhibition assay and an *in vivo* delayed-type hypersensitivity response. In their *in vitro* studies, soluble freeze-thawed extracts of *N. fowleri* were added to peripheral blood lymphocytes of guinea-pigs that had been immunized with heterologous and homologous *Naegleria* antigens. Conditioned media collected from the lymphocyte cultures, and containing elicited soluble factors, were used to test the response of macrophages. Macrophage migration was inhibited by migration inhibitory factor (MIF) obtained from the *Naegleria* antigen-sensitized lymphocytes. The authors suggested that exposure to *Naegleria* antigens elicits CMI, although exposure to homologous antigens was more efficient than exposure to heterologous antigens in inhibiting macrophage migration. In contrast, other studies using mice suggest that CMI does not play a role in protection against *Naegleria*. Newsome & Arnold (1985) demonstrated

that congenitally athymic mice (T cell deficient) and euthymic mice were equally susceptible to infection by *N. fowleri*. In addition, failure to impair host resistance to *N. fowleri* infection with diethylstilbestrol, which depresses delayed typed hypersensitivity, led Reilly *et al.* (1983b) to conclude that CMI alone does not constitute a major line of defense against *N. fowleri*. Thus, as in the case of humoral immunity, the role of CMI in protecting against infection by *Naegleria* remains unclear.

***Naegleria*–bacteria interactions**

In the environment, *Naegleria* are voracious feeders on bacteria, yeast, and algae. In seeking these food sources, *Naegleria* trophozoites transform into swimming flagellates. Preston & King (2003) demonstrated that *Naegleria* flagellates migrate from the soil layer to the water–air interface, dock at that site, and transform back into trophozoites in order to feed on bacteria that are present in abundance on the surface microlayer. Marciano-Cabral & Cline (1987) indicated that live *Escherichia coli* (*E. coli*) elicit a chemotactic response (directed migration) on the part of *Naegleria*, while on the other hand, *Naegleria* move away from toxin-producing bacteria and encyst. The growth and survival of amoebae in the presence of bacteria appear to vary with the density and species of bacteria.

The interaction of bacteria with amoebae can result in destruction of the bacteria, destruction of the amoebae, or development of a symbiotic relationship. *Naegleria* ingest bacteria by phagocytosis and by the use of ‘food-cups’ (Marciano-Cabral, 2004). Once ingested, proteins termed naegleriapores (*Naegleria* pore-forming proteins) lyse and degrade edible bacteria (Herbst *et al.*, 2002; Leippe & Herbst, 2004). Three strains of *Bacillus licheniformis* that have been isolated from the same soil habitat as *N. fowleri*, however, have been shown to exhibit amoebicidal activity (Galvez *et al.*, 1993). Symbiotic relationships have also been reported for *Naegleria* spp. Michel *et al.* (1999) and Walochnik *et al.* (2005) isolated a *Naegleria* strain that harbored two different populations of bacterial endocytobionts. Microscopic examination revealed a different localization and replication site for the two populations of bacterial endocytobionts that were present – one in the nucleus and the other in the cytoplasm. *Naegleria* may also serve as natural reservoirs for pathogenic bacteria. Early studies by Rowbotham (1980) suggested that free-living amoebae served as hosts for *Legionella*, the causative agent of Legionnaires’ disease. These observations are not surprising because in the environment amoebae and bacteria occupy the same niche including humidifier systems, cooling towers, and evaporative condensers that operate at mutual optimal growth temperatures. Barbaree *et al.* (1986) isolated *Naegleria* sp. and *Tetrahymena* sp. from cooling tower water that also

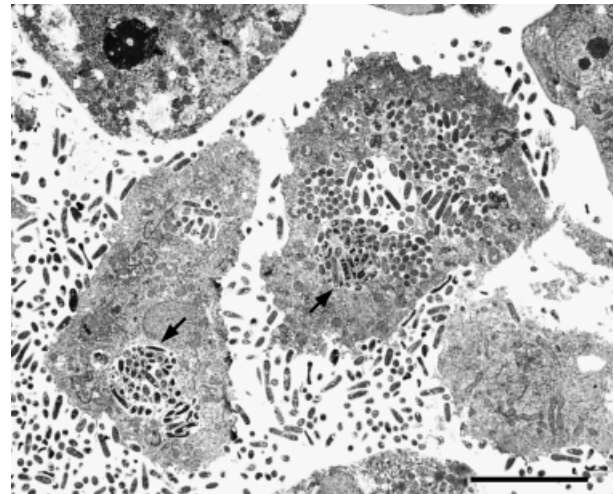


Fig. 8. Transmission electron micrograph depicting the uptake of bacteria by *Naegleria fowleri*. Trophozoites cocultured with *Legionella pneumophila* harbor numerous vesicular inclusions (arrow) replete with bacteria. The scale bar represents 10 μm .

contained *Legionella pneumophila* (*L. pneumophila*) and that was identified as a source of a legionellosis outbreak. Both protozoan isolates were shown to support the growth of *L. pneumophila* in laboratory culture. Rowbotham (1980) suggested that human infection with *Legionella* was acquired not by inhalation of free legionellae, but by inhalation of vesicles released from amoebae filled with legionellae or by inhalation of amoebae harboring bacteria (Fig. 8). In addition, cell culture models have been used to investigate the interaction of free-living amoebae with bacterial pathogens. *Naegleria* spp. were shown to support the growth of *Vibrio cholerae* (Thom *et al.*, 1992) and *L. pneumophila* (Newsome *et al.*, 1985). Newsome *et al.* (1985) studied the growth characteristics and interaction of *N. fowleri* with *L. pneumophila* by light and electron microscopy. Transmission electron microscopy revealed that the bacteria apparently underwent binary fission in cytoplasmic vacuoles within the amoebae. Dark-field microscopy also demonstrated that extracellular bacteria were found free in the culture media even after 6 days of coculture. However, the growth of bacteria within amoebae was observed to be contingent upon experimental culture conditions. *Naegleria fowleri* supported the growth of *Legionella* in amoebae growth medium but not in amoeba saline solution. In fact, when in saline solution, *Legionella* destroyed the amoebae (Newsome *et al.*, 1985). Rowbotham (1980, 1986) also reported that bacteria lysed amoebae when they were cultured on agar plates. In an attempt to simulate natural environmental conditions, Declerck *et al.* (2005) examined the influence of various bacteria on the uptake and intracellular replication of *L. pneumophila* by the nonpathogenic thermophilic species *N. lovaniensis*. The uptake and replication in *Naegleria* of

L. pneumophila serogroup 1, the most pathogenic strain of *Legionella*, was compared with that in another free-living amoeba, *Acanthamoeba castellanii*. It was found that, while other bacterial species commonly found with *L. pneumophila* in biofilms did not act as competitors in the uptake of *L. pneumophila*, they nevertheless influenced the replication of bacteria within the amoebae. Microscopic examination of cocultures revealed that 100% of *Acanthamoeba* were infected with *L. pneumophila*, whereas only 2% of *Naegleria* were infected with bacteria. However, *N. lovaniensis* formed cysts containing viable *L. pneumophila* while *A. castellanii* did not and was lysed by the bacteria. Declerck *et al.* (2005) suggested that encysted *Naegleria* containing *Legionella* could resist adverse conditions such as chlorination and biocidal compounds, an outcome that could account for their persistence in chemically treated sewage and water systems. Thus, it is now recognized that bacteria, either as endosymbionts or 'passengers,' are associated with free-living amoebae, including *Naegleria*. This interaction has potential major human health implications because bacteria residing in trophozoites or amoebic cysts are protected from biocides may be recalcitrant to antibiotic treatment, and by virtue of passage through amoebae may develop greater pathogenic capability.

Summary and conclusion

The number of reported cases of PAM has increased worldwide in recent years since *Naegleria fowleri*, the causative free-living amoeba of this fatal infection, was first recognized in 1965. The increased incidence may be due to greater awareness of the disease or due to the development of more rapid, highly sensitive, and specific diagnostic assays such as PCR. In addition, changes in environmental conditions, thermal pollution of water from industry, and the development of industrialized areas with nuclear power plants and cooling towers that allow for concomitant greater growth of amoeba and their bacterial food source may afford greater opportunities for infection. Indeed, recreational areas such as water parks, pools, and spas that are not properly maintained have been implicated as sources of human infections. Not all humans exposed to *N. fowleri* develop a fatal infection. However, the circumstances under which this free-living amoeba gains access to the human host and becomes highly pathogenic remain to be defined. Both pathogenic and nonpathogenic species of *Naegleria* destroy mammalian cells in culture under appropriate conditions. For nonpathogenic species, a high multiplicity of infection at 37 °C is required for such activity. Thus, cell culture does not distinguish pathogenic from nonpathogenic environmental isolates. To date, few proteins have been purified from *N. fowleri*, and little is known about its metabolic pathways, and its genome is yet to be sequenced. Immuniza-

tion with freeze-thawed amoebic extracts, administration of sublethal doses of amoebae, and passive transfer of immune serum or monoclonal antibodies have failed to provide protection against challenge infection in experimental animals. The innate immune system appears to play a critical role in protection against infection in which complement activity may constitute a core host defense element along with neutrophils and macrophages that mediate killing of trophozoites. Although pathogenic mechanisms remain to be defined, it is apparent that *N. fowleri* produce pore-forming proteins that lyse mammalian cells on contact, secrete proteases and phospholipases that degrade mammalian tissue, and synthesize regulatory surface proteins that confer protection to amoebae against complement-mediated lysis and other cytotoxic substances. Pathogenic *N. fowleri* exhibit a more rapid rate of locomotion in the presence of nerve cells and nerve cell products, and a more rapid rate of division as compared with nonpathogenic *Naegleria*. Collectively, their ability to evade the host immune system and to migrate rapidly in the host may contribute to their pathogenicity. The medical significance of free-living amoebae such as *N. fowleri* should not be underestimated, not only because they are agents of human disease but also because they can serve as reservoirs for pathogenic bacteria and, in this capacity, may sequester bacteria from biocides in the environment and antibiotics in the host.

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Dedication

The review is dedicated to the memory of Drs A. Julio Martinez and Thomas Byers, pioneers in the study of free-living amoebae, who shared their knowledge willingly with their colleagues.

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