Introduction

Acanthamoeba is a protozoan pathogen that has gained increasing attention during the last few decades. This is due to its ability to produce serious, as well as fatal, human and animal infections. The life cycle of Acanthamoeba consists of two stages: a vegetative trophozoite stage and a dormant cyst stage. The trophozoites are normally in the range of 12–35 μm in diameter; however, the size varies significantly between isolates belonging to different genotypes. During the trophozoite stage, Acanthamoeba actively feeds on bacteria, algae, yeasts, small organic particles and human cells, and reproduces by binary fission. Under harsh conditions (i.e. lack of food, increased osmolarity or hypo-osmolarity, extremes in temperatures and pH, and high cell densities), trophozoites differentiate into cysts of 5–20 μm in diameter, but again this size varies between isolates belonging to different genotypes. Cysts are airborne, which may help spread Acanthamoeba in the environment and/or help it reach susceptible hosts. One reason that Acanthamoeba infections are notoriously difficult to treat is the rapid propensity of the trophozoites to transform into cysts, which are highly resistant to antimicrobial compounds. The infective trophozoites emerge from the cysts under favourable conditions, thus completing the life cycle (Khan, 2006; Marciano-Cabral & Cabral, 2003; Visvesvara et al., 2007). The genus Acanthamoeba consists of both pathogenic and non-pathogenic isolates. The pathogenic Acanthamoeba are now well-recognized to produce a painful, blinding keratitis that is mostly associated with contact lens wear and a fatal granulomatous encephalitis in immunocompromised or debilitated individuals. These infections are of major concern in view of: (i) increasing numbers of immunocompromised persons, (ii) increasing numbers of individuals undergoing immunosuppressive therapy and excessive use of steroids, and (iii) global warming, which may add to the ubiquity of these pathogens, and thus a possibility of increased exposure to the susceptible hosts. With the growing human immunodeficiency virus pandemic, it is reasonable to predict a further increase in the numbers of Acanthamoeba encephalitis cases. Perhaps, the most distressing aspect is the limited availability of effective treatment against Acanthamoeba encephalitis, with a case fatality rate of more than 90%. The gross pathology of the autopsied brain often shows severe oedema and haemorrhagic necrosis with severe meningeal irritation and encephalitis. The microscopic findings of the post-mortem biopsies reveal amoebae cysts, predominantly in the perivascular spaces in the parenchyma, indicating the involvement of the cerebral capillaries as the sites of entry into the central nervous system (CNS). The purpose of this review is to present the current understanding of Acanthamoeba penetration of the blood–brain barrier, a key step in parasite invasion of the CNS.

Neurological aspects of Acanthamoeba encephalitis

Magnetic resonance imaging and computed tomography of the Acanthamoeba encephalitis brain show ring-enhancing lesions or low-density abnormalities mimicking a single or multiple space-occupying mass, respectively. Depending on the location of the lesions, Acanthamoeba encephalitis exhibits a broad spectrum of neurological signs and symptoms. In the majority of cases, lesions are most numerous in the temporal and parietal lobes. However, severely immunocompromised patients may not exhibit such lesions (Martinez, 1985, 1991; Martinez & Visvesvara, 1997). Neurological manifestations vary, and may include headache, fever, behavioural changes, hemiparesis, lethargy, stiff neck, agitation, aphasia, ataxia, vomiting, nausea,
cranial nerve palsies and increased intracranial pressure (Martinez, 1985, 1991; Martinez & Visvesvara, 1997). For simplicity, the signs determined by neurological examination can be grouped into four categories as follows:

(i) state of consciousness – this may range from confused to being unconscious,
(ii) loss of reflex activity – this may be observed for part or all of body or in the function of organs, as well as internal functions,
(iii) abnormal speech patterns, e.g. aphasia,
(iv) abnormal motor patterns – imbalance or unsteady gait or seizure.

Later stages involve a complete loss of consciousness, seizures and coma, which are poor prognostic signs, with most patients who reach this level of neurological deficit deteriorating and dying within days. The pathophysiological complications involving the CNS most likely include induction of pro-inflammatory responses, invasion of the blood–brain barrier and neuronal damage leading to the brain dysfunction (Benedetto & Auriault, 2002a, b; Khan, 2006, 2007; Marciano-Cabral & Cabral, 2003; Marciano-Cabral et al., 2004; Mattana et al., 2002; Shin et al., 2001; Visvesvara et al., 2007).

Routes of entry into the CNS

There are two possible routes of entry to the CNS: through the nasal passage or via the blood. In humans, Acanthamoeba spp. are usually blood-borne while other free-living amoeba, such as Naegleria spp., enter through the nasal passages (Cerva, 1967; Culbertson et al., 1959). It is widely accepted that the routes of entry for Acanthamoeba include the respiratory tract, leading to amoebae invasion of the alveolar blood vessels, followed by haematogenous spread. Skin lesions may provide direct entry into the bloodstream, bypassing the lower respiratory tract (Fig. 1). Acanthamoeba entry into the CNS most likely occurs through the blood–brain barrier (Martinez, 1985, 1991), which is highly selective in regulating the entry of microbes/molecules. The olfactory neuroepithelium route (i.e. invasion of the olfactory part of the nasal epithelium, and migration along nerve fibres, followed by invasion of the olfactory bulb) provides another route of entry into the CNS and has been studied in experimental models (Martinez, 1991; Martinez & Visvesvara, 1997). It is worth noting that the olfactory part of the nasal mucosa in the rat (and other rodents) is 50 % of the total surface area of the nose, while in humans the olfactory part of the nasal mucosa is only 3–8 % (Graff & Pollack, 2005; Illum, 2000). The smaller fraction of nasal mucosa associated with olfactory epithelium in humans can be associated with a lower likelihood of this route leading to human disease. Furthermore, the widespread distribution of lesions in the brain would fit better with haematogenous spread than olfactory access. Amoebae entry into the CNS most likely occurs through the endothelial lining of cerebral capillaries (Martinez et al., 1975; Martinez, 1985, 1991). In support, haematoxylin- and eosin-stained sections of the brain tissue of Acanthamoeba encephalitis patients exhibit large numbers of amoebae in the perivascular space. Pathogens that enter via the choroid plexus route end up in the cerebrospinal fluid (CSF). Affected tissues other than the CNS may include the subcutaneous tissue, skin, liver, lungs, kidneys, adrenal glands, pancreas, prostate, lymph nodes and bone marrow, which further suggest haematogenous spread. However, the CSF is continuous with the extracellular fluid of the brain parenchyma, and flows...
unidirectionally from ventricles down to the lumbar region, thus spinal taps of patients may aid in the early diagnosis, which is a pre-requisite in the successful treatment of *Acanthamoeba* encephalitis (Petry et al., 2006).

**Blood–brain barrier**

Blood vessels serving the brain can be collectively called the blood–brain barrier. There are more than 100 billion capillaries in the human brain extending ~400 miles with a surface area of 20 m² providing an extensive network fulfilling the nutrient demands as well as maintaining homeostasis of the CNS (Pardridge, 2002). Two different structures separate the blood from the CNS: the blood–brain barrier and the blood–CSF barrier. The blood–brain barrier includes the endothelial lining of the brain capillaries, which are closely associated with pericytes, astrocytes and neurones (Fig. 2). The presence of astrocytes ensheathing the endothelium is limited to the blood–brain barrier. Astrocytes are involved in the induction of the permeability-barrier properties of the blood–brain barrier. Pericytes are also closely associated with the endothelial cells and are required for capillary maturation, while basement membrane [containing laminin, collagen IV, proteoglycans (especially heparan sulphate), fibronectins, nidogen and entactin], is important for the blood–brain barrier differentiation. In addition to cellular differences, the blood–brain barrier is characterized by the presence of tight junctions (Fig. 2). The tight junctions are primarily responsible for the barrier function preventing the entry of blood contents into the CNS, making it a highly selective barrier compared with the peripheral endothelium (Miller, 1999). For comparison, endothelial cells of the blood–brain barrier exhibit high transendothelial electrical resistance (~2000 Ω cm⁻²) (Pardridge, 1999), while endothelial cells from human placenta exhibit <50 Ω cm⁻² (Jinga et al., 2000). The presence of tight junctions in the blood–brain barrier ensures that even small molecules, such as dyes and antibiotics, are prevented from entry into the CNS, by the limiting of the paracellular route. In contrast, non-brain endothelium is more permissive. However, small lipophilic molecules, such as O₂ and CO₂, can diffuse freely across the plasma membrane along their concentration gradient. Nutrients, such as glucose and amino acids, are transported across the blood–brain barrier via transporters, while larger molecules, such as insulin, leptin, iron and transferrin, are taken up via receptor-mediated endocytosis to support neuronal function. The junction complex in the blood–brain barrier comprises tight junctions and adherens junctions. The tight junctions consist of integral membrane proteins (i.e. claudin, occludin, junction adhesion molecule, endothelial cell-selective adhesion molecules) and cytoplasmic proteins [zonula occludens (ZO) proteins ZO-1, ZO-2, ZO-3]. Claudins bind to claudins on the adjacent endothelial cells to form tight junctions. The carboxy terminal of claudins binds to cytoplasmic ZO proteins. Claudin-5 and claudin-3 are localized in the tight junctions of brain endothelial cells. Occludin is a phosphoprotein and its cytoplasmic domain is directly associated with ZO proteins. Junction adhesion molecules and endothelial cell-selective adhesion molecules also are localized in tight

**Fig. 2.** Schematic illustration of the blood–brain barrier at the cerebral capillary endothelium exhibiting tight junctions. The endothelial cells are surrounded by basement membrane, which is ensheathed by astrocytes and pericytes. The tight junctions are composed of integral proteins, including occludin, claudin, junction adhesion molecule (JAM) and endothelial cell-selective adhesion molecule (ECSAM), that interact with their counterparts on the adjacent endothelial cells. Both tight junctions and adherens junctions are composed of multiple proteins. The cytoplasmic tails of these proteins interact with the actin cytoskeleton via a number of accessory proteins including members of the zonula occludens (ZO). VE, vascular endothelium.
junctons of the blood–brain barrier. The accessory proteins of the ZO provide structural support and bind to integral membrane proteins of the tight junctions, further linking membrane proteins to the actin cytoskeleton (Fig. 2) (Huber et al., 2001; Kim et al., 2006; Tuomanen, 1996). In contrast, adherens junctions are composed of membrane protein involving cell-to-cell cadherin homotypic interactions and cadherin–catenin interactions. Adherens junction proteins include cadherin, actinin and vinculin (the analogue of catenin).

Factors contributing to Acanthamoeba entry into the CNS

The mechanism by which Acanthamoeba traverses the blood–brain barrier is complex and is likely to involve both parasite (adhesins, proteases, phospholipases) as well as host determinants [interleukin beta (IL–β), IL–α, tumour necrosis factor alpha (TNF–α), gamma interferon (IFN–γ), host cell apoptosis]. The overall outcome is increased permeability and/or apoptosis of the brain endothelial cells, which promotes blood–brain barrier disruptions leading to parasite invasion of the CNS. The understanding of the cross-talk between parasite–host interactions, as well as between the blood–brain barrier and the CNS, in this disease will provide insights to its neuropathogenesis and may help the development of novel therapeutic interventions.

Parasite factors (contact-dependent and -independent mechanisms)

The availability of in vitro cultures of primary human brain microvascular endothelial cells (HBMEC) has been of tremendous value in the identification and characterization of molecular determinants that are required in host–parasite interactions (Alsam et al., 2003; Stins et al., 1997). The HBMEC used in my laboratory are positive for brain endothelial markers such as factor VIII, carbonic anhydrase, Ulex europaeus agglutinin I and gamma-glutamyl transpeptidase, and took up acetylated low-density lipoprotein. In addition, the HBMEC demonstrated the expression of tight junction proteins and formation of a polar monolayer. Using HBMEC, clinical isolates of Acanthamoeba were shown to bind to HBMEC. Interestingly, non-pathogenic environmental isolates exhibit minimal binding to HBMEC, suggesting that adhesion to HBMEC is an important step in parasite invasion of the blood–brain barrier (Alsam et al., 2003). The adhesion is mediated by a mannose-binding protein (MBP) expressed on the surface membranes of pathogenic isolates of Acanthamoeba (Alsam et al., 2003; Garate et al., 2004). At present, the identity of the MBP receptor in the host endothelial cells is unknown and requires further investigation. However, parasite adhesion to the host cells induces tyrosine phosphorylation of several HBMEC proteins (unpublished findings). Recent studies have shown that Acanthamoeba binding to the surface of HBMEC causes activation of Rho-associated intracellular signalling cascades (unpublished findings). Rho-associated pathways could disturb the function of tight junctions, thus leading to increased blood–brain barrier permeability (Fig. 3). For example, recent studies have shown that RhoA regulates myosin light-chain phosphorylation, causing structural changes and redistribution of ZO-1 and occludin, leading to increased blood–brain barrier permeability (Shen et al., 2006). Continued incubations resulted in HBMEC apoptosis (Sissons et al., 2005). Using LY294002, a specific phosphatidylinositol 3-kinase (PI3K) inhibitor, as well as using HBMEC expressing mutant p85, i.e. a regulatory subunit of PI3K (dominant-negative PI3K), it has recently been shown that PI3K plays a crucial role in Acanthamoeba-mediated HBMEC death (Sissons et al., 2005). Other studies have shown that Acanthamoeba induced cell cycle arrest by upregulating the expression of genes such as those encoding GADD45A and p130Rb, which are associated with cell cycle arrest, as well as inhibiting the expression of other genes, such as those encoding cyclins F, G1 and cyclin-dependent kinase-6 proteins, important for cell cycle progression (Sissons et al., 2004). The overall response of these events is shown to be arrest of the HBMEC cell cycle. This is further supported by the dephosphorylation of retinoblastoma protein, a potent inhibitor of G1/S cell cycle progression (Sissons et al., 2004).

In vivo, endothelial cells that are part of the fully formed blood vessels are not always replicating compared to growing endothelial cell lines. Thus the loss of cell cycle control implies that adaptive mechanisms that maintain homeostasis or haemostasis may not be induced, and these aspects are being investigated. Overall, for a complete understanding of the molecular mechanisms involved in amoeba-mediated cell cycle arrest and induction of amoeba-mediated HBMEC apoptosis further investigation is needed. Additionally, it is not clear whether these pathways are linked or independent of each other. Additionally, phagocytosis contributes to Acanthamoeba-mediated host cell damage. The ability of Acanthamoeba to form food cups or amoebastomes during incubations with host cells suggests that phagocytosis plays an important role in Acanthamoeba pathogenesis (Alsam et al., 2005a; Khan, 2001; Pettit et al., 1996). In support of this, it is shown that cytochalasin D (a toxin that blocks actin polymerization) inhibits Acanthamoeba-mediated HBMEC death, confirming that actin-mediated cytoskeletal rearrangements of Acanthamoeba are important to their ability to phagocytose HBMEC (unpublished findings). Overall, these studies showed that Acanthamoeba MBP-mediated binding to HBMEC results in host–parasite cross-talk that involves events both at the transcriptional- and post-translational levels. These pathways result in HBMEC dysfunction, which may assist amoebal invasion of the blood–brain barrier. Among contact-independent mechanisms, extracellular serine proteases are seen to be the most potent in
amoebae-mediated HBMEC monolayer disruptions (Alsam et al., 2005b). In addition, Acanthamoeba serine proteases have been shown to degrade types I, III and IV collagen, elastin and fibronectin, which are the main components of the extracellular matrix, as well as fibrinogen, IgG, IgA, albumin, plasminogen (involved in proteolytic degradation of the extracellular matrix) and haemoglobin (Kong et al., 2000; Sissons et al., 2006). This highlights a role for extracellular proteases in facilitating the migration of Acanthamoeba from the systemic circulation into the interior of the brain. Acanthamoeba proteases are produced constitutively, but are upregulated in response to binding to HBMEC (Alsam et al., 2005b). Of note, Acanthamoeba proteases disrupt HBMEC monolayers, but do not appear to induce HBMEC cytotoxicity as determined by lactate dehydrogenase release (Alsam et al., 2005b; Sissons et al., 2006). These findings suggest that proteases induce blood–brain barrier permeability, most likely by targeting tight junctions. In support, recent studies have shown that Acanthamoeba proteases target ZO-1 and occludin, major components of tight junctions (Fig. 3) (Khan, 2007).

Host factors (immune-mediated mechanisms)
Acanthamoeba is an extracellular pathogen and is directly exposed to the host immune system. The immune response plays an active role in protection against this pathogen, as well as contributing to blood–brain barrier perturbations and disease development. For example, characteristic granulomatous lesions in the CNS are a result of the host immune response and are most likely composed of CD4 and CD8 T cells, B lymphocytes, plasma cells, macrophages and parasites. The localization of immune cells in the brain suggests the involvement of pro-inflammatory cytokines in protection as well as in pathophysiological complications. IFN-γ is one of the earliest cytokines to be involved and may play an important role in the activation of immune cells. IFN-γ, through the pro-inflammatory network, upregulates the release of specific cytokines, including TNF-α, IL-6, IL-β and IL-1α, which may initiate the immune response to Acanthamoeba in the brain (Benedetto et al., 2003). Other studies showed that microglial cells secrete IL-β, IL-α and TNF-α in response to the parasite (Marciano-Cabral et al., 2000). Microglia primed with IFN-γ and TNF-α exhibit amoebicidal effects (Benedetto &
Auriault, 2002a, b). In contrast, microglia primed with IFN-γ and IL-6 exhibit amoebistatic effects (Benedetto & Auriault, 2002a, b). The ability of microglia to exhibit antimicrobial properties is most likely mediated by the generation of free radicals, i.e. nitric oxide (NO), and production of pro-inflammatory and anti-inflammatory cytokines. However, overproduction of NO induces toxic effects and may contribute to pathological disorders. For example, NO acts at the synapse level inhibiting N-methyl-D-aspartic acid Ca2+ channels, preventing the influx of Ca2+ into the neurone and generating focal damage or inducing apoptosis in the brain inflammatory cells. Overall, one of the key factors in the disease state is a result of the imbalance in cytokine levels, i.e. overproduction of pro-inflammatory cytokines or deficiency of host-protective mechanisms, mediated by anti-inflammatory molecules, resulting in immunosuppression, which may determine the susceptibility to and severity of Acanthamoeba encephalitis. However, an understanding of the complex interactions of cytokines with the immune cells and how they inflict irreplaceable brain damage need further investigation.

New approaches for drug development

In Acanthamoeba infection the involvement of the CNS almost always results in death. This is due to the unavailability of effective and/or recommended drugs, and the inability of drugs to traverse the blood–brain barrier to gain entry into the brain to kill parasites. Current treatment usually involves a combination drug approach, given intravenously, including various drugs such as ketoconazole, fluconazole, sulfadiazine, pentamidine isethionate, amphotericin B, azithromycin, itraconazole or rifampicin, which rarely leads to a successful prognosis, and heavily depends on early diagnosis, followed by aggressive treatment. Thus, there is an urgent need to initiate both drug discovery and drug delivery programmes. Currently, the delivery system for the CNS drugs may include: (i) trans-cranial drug delivery to the brain, i.e. drug delivery to the ventricles of the brain by injection through a trephine hole in the cranium (intracerebroventricular injection, intracerebral injection or convection-enhanced diffusion), (ii) trans-nasal drug delivery to the brain, a method that mimics the current in vivo Acanthamoeba encephalitis model in mice (lipid-soluble small molecules may be instilled nasally, cross the nasal mucosa and the arachnoid membrane, and enter the olfactory CSF with a result similar to intracerebroventricular injection) and (iii) transient blood–brain barrier disruption (arterial infusion of hyperosmotic solution or ultrasonic irradiation of the brain). All of the above methods may have complications due to side effects. Other approaches may include lipidization of small molecules to enhance transport across the blood–brain barrier, carrier-mediated transport of drugs, or administration of non-viral plasmid DNA encoding antisense RNA against the virulence genes of Acanthamoeba.

Conclusion

The burden of Acanthamoeba encephalitis is not truly appreciated. This is due to the lack of awareness, difficulty in diagnosis, and most distressingly the lack of an effective treatment. A vast number of Acanthamoeba encephalitis cases have most likely been undetected, and the actual number of cases must be significantly higher (Khan, 2006). For example, there is no single report of Acanthamoeba encephalitis in Africa, despite the presence of millions of human immunodeficiency virus-infected individuals, who are susceptible hosts for opportunistic pathogens, and live in a warm climate, where there is probable frequent environmental exposure and subordinate sanitation in many places. Acanthamoeba invasion of the CNS most likely occurs at the blood–brain barrier. However, it is far from clear how the aforementioned determinants contribute to Acanthamoeba traversal of the blood–brain barrier. The pathophysiological complications involving the CNS most likely include induction of the pro-inflammatory responses, and invasion of the blood–brain barrier and the connective tissue leading to neuropathogenesis. A better understanding of parasite–host cell interactions that are involved in Acanthamoeba penetration of the blood–brain barrier should help in the development of therapeutic interventions and/or the design of new strategies for disease prevention.

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