Acanthamoeba can cause fatal granulomatous amoebic encephalitis (GAE) and eye keratitis. However, the pathogenesis and pathophysiology of these emerging diseases remain unclear. In this study, the effects of Acanthamoeba on the host cell cycle using human brain microvascular endothelial cells (HBMEC) and human corneal epithelial cells (HCEC) were determined. Two isolates of Acanthamoeba belonging to the T1 genotype (GAE isolate) and T4 genotype (keratitis isolate) were used, which showed severe cytotoxicity on HBMEC and HCEC, respectively. No tissue specificity was observed in their ability to exhibit binding to the host cells. To determine the effects of Acanthamoeba on the host cell cycle, a cell-cycle-specific gene array was used. This screened for 96 genes specific for host cell-cycle regulation. It was observed that Acanthamoeba inhibited expression of genes encoding cyclins F and G1 and cyclin-dependent kinase 6, which are proteins important for cell-cycle progression. Moreover, upregulation was observed of the expression of genes such as GADD45A and p130 Rb, associated with cell-cycle arrest, indicating cell-cycle inhibition. Next, the effect of Acanthamoeba on retinoblastoma protein (pRb) phosphorylation was determined. pRb is a potent inhibitor of G1-to-S cell-cycle progression; however, its function is inhibited upon phosphorylation, allowing progression into S phase. Western blotting revealed that Acanthamoeba abolished pRb phosphorylation leading to cell-cycle arrest at the G1-to-S transition. Taken together, these studies demonstrated for the first time that Acanthamoeba inhibits the host cell cycle at the transcriptional level, as well as by modulating pRb phosphorylation using host cell-signalling mechanisms. A complete understanding of Acanthamoeba–host cell interactions may help in developing novel strategies to treat Acanthamoeba infections.

INTRODUCTION

Acanthamoeba are the causative agents of life-threatening granulomatous amoebic encephalitis (GAE) in immunocompromised patients (Jones et al., 1975; Martinez, 1987; Di Gregorio et al., 1992; Friedland et al., 1992; Gonzalez et al., 1986; Gordon et al., 1992) and a common amoebic keratitis, a sight-threatening disease of the eye, which is mostly associated with contact-lens use (Chynn et al., 1995; Moore & McCulley, 1989; Wright et al., 1985; Mathers et al., 1996; Niederkorn et al., 1999; Marciano-Cabral & Cabral, 2003; Khan, 2003). In addition, Acanthamoeba have been associated with cutaneous lesions and sinusitis in immunocompromised patients (reviewed by Marciano-Cabral & Cabral, 2003). Over the past few decades, Acanthamoeba infections have remained significant and are on the rise. This is due to increasing populations of contact-lens wearers and immunocompromised patients, as well as improved awareness and detection of these emerging diseases. However, the precise mechanisms associated with the pathogenesis and pathophysiology of Acanthamoeba infection remain unclear. The clinical outcome of Acanthamoeba infection is dependent on the virulence of the parasites, the hosts and environmental factors (Khan, 2003). Given their complexity, it is not surprising that Acanthamoeba interactions with host cells result in the stimulation of diverse signalling pathways, which ultimately results in the well-documented Acanthamoeba-produced host cell cytotoxicity (Khan, 2003; Cao et al., 1998; Leher et al., 1998; De Jonckheere, 1983). Other studies have shown that Acanthamoeba produces apoptosis in murine neuroblastoma cells (Alizadeh et al., 1994). Apoptosis, or programmed cell death, is known to be dependent on host cell signalling. Overall, these studies suggested that interactions of Acanthamoeba with host cells stimulated specific host cell signalling pathways, resulting in...
host cell death. We hypothesized that *Acanthamoeba* could influence the proliferation of host cells, as well as inducing host cell apoptosis. In this study, we determined the effects of *Acanthamoeba* on the host cell cycle. Using cell-cycle-specific gene array analyses, we determined that *Acanthamoeba* inhibited DNA synthesis, inducing host cell-cycle arrest, one of the earliest events in *Acanthamoeba*-produced host cell apoptosis/cytotoxicity. These data were further confirmed at the protein level by studying the phosphorylation of the retinoblastoma protein (pRb), a master regulator of the cell cycle. Hyperphosphorylation of pRb is crucial for cell-cycle progression from G1 to S phase. We showed that *Acanthamoeba* induced pRb dephosphorylation in host cells resulting in G1-to-S phase checkpoint arrest. This is the first report to show that *Acanthamoeba* induces host cell-cycle arrest.

**METHODS**

All chemicals were purchased from Sigma, unless otherwise stated.

*Acanthamoeba* cultures. Two pathogenic *Acanthamoeba* isolates were used in this study. The first, belonging to the T1 genotype, was isolated from a GAE case (ATCC 50494) and the second, belonging to the T4 genotype, was isolated from a keratitis case (ATCC 50492). Parasites were routinely grown in PYG medium [0-75 % (w/v) proteose peptone, 0-75 % (w/v) yeast extract and 1-5 % (w/v) glucose] at 30 °C in tissue culture flasks and the medium was refreshed 17-20 h prior to use. All chemicals were purchased from Sigma, unless otherwise stated.

Human brain microvascular endothelial cells (HBMEC). Primary HBMEC from a human origin were isolated and cultured as described previously (Alsam et al., 2003). Briefly, HBMEC were purified by fluorescence-activated cell sorting and their purity was tested using endothelial markers such as expression of F-VIII, von Willebrand factor, and uptake of acetylated low-density lipoprotein, resulting in >99 % pure cultures. HBMEC were routinely grown on rat tail collagen-coated dishes in RPMI 1640 containing 10 % fetal bovine serum, 10 % Nunlemogen, 2 mM glutamine, 1 mM pyruvate, 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹, non-essential amino acids and vitamins (Alsam et al., 2003).

Human corneal epithelial cells (HCEC). Immortalized HCEC were routinely cultured as described previously (Araki-Sasaki et al., 2000). Briefly, HCEC were grown in RPMI 1640 containing 10 % fetal calf serum and 2 mM glutamine. Under these conditions, HCEC exhibited human corneal epithelial cell-specific properties, as described previously (Araki-Sasaki et al., 2000). For adhesion and cytotoxicity assays, both HBMEC and HCEC were grown in 24-well plates by incubating 10⁶ cells per well. For gene array and Western blotting assays, HBMEC and HCEC were grown in 60 mm dishes at a density of 3 × 10⁵ cells per well.

Cytotoxicity assay. To determine the pathogenic potential of each isolate used in this study, cytotoxicity assays were performed as described previously (Khan, 2001). Briefly, both HBMEC and HCEC were grown to 95 % confluence in RPMI 1640. Cells were incubated at 37 °C in tissue culture flasks and the medium was refreshed 17-20 h prior to experiments, as described previously (Khan et al., 2000). This resulted in >99 % of *Acanthamoeba* in trophozoite forms.

To test the effect of saccharides, amoebae (2 × 10⁵) were incubated with various saccharides [α-D-mannopyranoside (α-mannose), xylose, α-L-fucose and β-D-galactose, all at a final concentration of 100 mM] in 100 μl RPMI 1640 for 30 min prior to the adhesion assay. For controls, amoebae were incubated with BSA.

**Cell-cycle-specific gene array.** To determine whether *Acanthamoeba* altered cell-cycle-related gene expression, a cell-cycle-specific gene array was employed (Superarray) according to the manufacturer’s instructions. Briefly, both HBMEC and HCEC were grown to 95 % confluence in 60 mm plates and incubated with *Acanthamoeba* trophozoites (5 × 10⁴ per plate). The GAE isolate was incubated with HBMEC, while the keratitis isolate was incubated with HCEC. Plates were incubated at 37 °C in 5 % CO₂ for 30 min. For controls, HBMEC and HCEC were incubated alone under similar conditions. Following this incubation, cells were washed three times with PBS-Ca and RNA was isolated using an RNAqueous kit (Ambion) according to the manufacturer’s instructions. RNA purity and quality was determined by measuring absorbance at 260 and 280 nm using a spectrophotometer and confirmed by RNA agarose gel analysis (Khan et al., 2003).

For array analyses, 5 μg RNA was converted to cDNA using a primer mix (Superarray) specific for 96 cell-cycle-specific genes (the list of genes tested in this study is available at http://www.superarray.com/gene_array_table/spd_HS-001_table.pdf). Reactions were carried out using the Moloney murine leukaemia virus reverse transcriptase and biotin-16-UTP (Promega) to produce a biotinylated cDNA probe. cDNA probes from infected and uninfected cells were hybridized with separate membranes overnight at 60 °C. The membranes were washed twice with 5 ml wash buffer A (2 × SSC, 1 % SDS) at 60 °C for 5 min and twice with wash buffer B (0-1 × SSC, 0-5 % SDS) at 60 °C for 5 min, according to the manufacturer’s instructions. The cDNA probe was detected using 1 ml CDP-Star detection solution (Superarray). Images generated by scanning were analysed using SCANALYZE software (available at http://rana.Stanford.edu/software) to determine the approximate fold change for each spot on the array following normalization of control genes on the two arrays (GEArray Analyser; Superarray). Levels of gene expression of more than 2-fold and < 0-3-fold were considered to be significantly up- or downregulated, respectively, according to the manufacturer’s instructions.

**Western blotting.** Western blotting was performed to determine pRb phosphorylation as described previously (Khan et al., 2002). Briefly, HBMEC and HCEC were grown as monolayers in 60 mm dishes and the cells were incubated with *Acanthamoeba* (approx. 5 × 10⁴) for various
Host cell-cycle arrest induced by Acanthamoeba

intervals of time. Unbound amoebae were removed by several washes and cells were lysed with RIPA lysis buffer (50 mM Tris/HCl, pH 7-4, 0-1 % SDS, 0-5 % sodium deoxycholate, 10 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 % Triton X-100, 1 mM Na3VO4, 50 mM NaF, 1 mM PMSF, 1 μg aprotonin ml⁻¹, 1 μg leupeptin ml⁻¹ and 1 μg pepstatin ml⁻¹). For immunoprecipitation, equal amounts of cell lysates (0-5–1 mg) were incubated with antibodies against phospho-Rb including the phospho-Rb Ser780, Ser795 and Ser807/811 residues (Cell Signalling Technology) overnight at 4 °C. Following this, 50 μl Protein A–agarose beads (Quagen) was added for 1 h to collect the antigen–antibody complex. Antigen–antibody complexes were washed three times and analysed by 7-5 % SDS-PAGE. Proteins were transferred onto a nitrocellulose membrane. Membranes were blocked in 4 % blocking solution (BioRad) in PBS-Ca and immunoblotted using a primary mAb against total pRb (Cell Signalling Technology) overnight at 4 °C. Next day, membranes were washed and subsequently incubated (60 min, 22 °C) with horseradish peroxidase-linked secondary antibody. In the controls, half of the cell lysates were immunoprecipitated and immunoblotted using anti-pRb antibody. A single band of antibody-bound pRb was visualized using an enhanced chemiluminescence kit (Amersham Biosciences).

RESULTS AND DISCUSSION

Acanthamoeba isolates belonging to genotypes T1 and T4 do not exhibit tissue specificity in host cell binding

Two clinical isolates of Acanthamoeba belonging to the T1 genotype (GAE isolate) and T4 genotype (keratitis isolate) were used and their binding determined using HBMEC and HCEC. We observed that both isolates exhibited more than 78 % binding to both HCEC and HBMEC. Furthermore, binding was significantly inhibited in the presence of exogenous α-mannose (P < 0.01), indicating the role of mannose-binding protein (MBP). It was interesting to note that >75 % of the binding of the keratitis isolate was blocked using α-mannose, irrespective of the cell type. α-Mannose inhibited approximately 50 % of the binding of the GAE isolate to both HCEC and HBMEC cell types, suggesting the role of other determinants in binding of the GAE isolate to host cells (Fig. 1).

Acanthamoeba isolates belonging to genotypes T1 and T4 exhibit severe host cell cytotoxicity

The pathogenic potential of the keratitis (T4) and GAE (T1) isolates was determined using HBMEC and HCEC. The T4 isolate produced 74 ± 3.5 and 80 ± 4.2 % cytotoxicity in HBMEC and HCEC, respectively. Similarly, the T1 isolate produced 80 ± 2.6 and 77 ± 5.6 % cytotoxicity in HBMEC and HCEC, respectively. Overall, these results indicated that both isolates are potential pathogens and exhibit severe cytotoxicity, irrespective of the cell type.

Gene array analyses demonstrate inhibition of the cell cycle

To determine the effects of Acanthamoeba on the cell cycle of HBMEC and HCEC, a cell-cycle-specific gene array was employed to screen for 96 genes important in cell-cycle regulation. We observed that Acanthamoeba induced significant changes in the expression of 18 genes encoding proteins that regulate cell-cycle progression in HBMEC (Table 1). These included genes such as cyclins D2, D3, F, G1, CDK6 and p130RB2, which encode proteins important for G1-to-S progression in the cell cycle (Gillett & Barnes, 1998; Pietenpol & Stewart, 2002; Poggio et al., 2002). Moreover, significant upregulation of the expression of genes such as GADD45 and CDC6 was observed. GADD45 is known to inhibit cyclin-dependent kinase-1 (CDK1) by physically dissociating the CDK1–cyclin B complex, eliminating CDK1 activity and resulting in G2-to-M checkpoint arrest (Zhan et al., 1999). Overall, our data showed that Acanthamoeba induced alterations in the expression of genes encoding proteins required for cell-cycle progression. Similar results were obtained with Acanthamoeba interactions with HCEC (data not shown).

Acanthamoeba inhibits pRb phosphorylation, inducing cell-cycle arrest at the G1-to-S checkpoint

To determine the significance of our findings at the protein level, we studied the effect of Acanthamoeba on the host cell pRb, a master regulator of the cell cycle (Harbour & Dean, 2000; Dyson, 1998; Stevaux & Dyson, 2002). pRb controls the G1-to-S transition of the cell cycle by directly associating with the transactivation domain of E2F and blocking the ability of E2F to activate transcription of genes (G1 arrest), which is required for G1-to-S phase progression. However, upon stimulation with growth signals, pRb becomes phosphorylated and dissociates from the E2F transcription factors, allowing E2F to act as a transcriptional activator. To
determine the effects of *Acanthamoeba* on pRb phosphorylation, HBMEC and HCEC were incubated with *Acanthamoeba* followed by immunoprecipitation and Western blotting using anti-phospho-pRb and anti-pRb antibodies, respectively. We observed that HBMEC and HCEC exhibited pRb dephosphorylation in response to *Acanthamoeba* (Fig. 2). This pRb dephosphorylation was observed in a time-dependent manner, indicating the role of host cell signalling. It was interesting to note that HCEC showed a similar pattern, but over a longer time period (Fig. 2b), and that this delayed response was observed with both T1 and T4 isolates (data not shown). Overall, our data showed that *Acanthamoeba*–host cell interaction abolishes pRb phosphorylation, resulting in cell-cycle arrest.

Several lines of evidence suggest that *Acanthamoeba* produces severe host cell cytotoxicity (Cao *et al.*, 1998; De Jonckheere, 1983; Khan, 2001; Leher *et al.*, 1998), which correlates with clinical findings of painful, blinding keratitis and a fatal encephalitis (GAE). However, the underlying mechanisms leading to the pathological features associated with *Acanthamoeba* infection remain unclear. In this study, we demonstrated that *Acanthamoeba* induces host cell-cycle arrest, a primary step in the pathogenesis of *Acanthamoeba*-mediated host cell cytotoxicity.

As indicated above, *Acanthamoeba* are known to produce two recognized diseases, GAE and keratitis. For biological relevance, we used a GAE isolate of *Acanthamoeba* belonging to the T1 genotype and studied its effect on HBMEC. Similarly, a keratitis isolate belonging to the T4 genotype was used for HCEC. We did not observe tissue specificity with *Acanthamoeba* binding to the host cells. This could be due the fact that the *Acanthamoeba* isolates tested in this study are capable of producing both diseases and/or that initial binding of *Acanthamoeba* to host cells requires merely the presence of mannose residues on the host cells. In support of the second hypothesis, previous reports have shown that *Acanthamoeba* exhibit binding to various cell types including rabbit corneal epithelial cells (Morton *et al.*, 1991; Yang *et al.*, 1997), pig corneal epithelium (van Klink *et al.*, 1992), Chinese hamster corneal epithelium (van Klink *et al.*, 1993), human corneal fibroblasts (Badenoch *et al.*, 1995) and rat microglial cells (Shin *et al.*, 2001), as well as to tissue.
culture plates coated with mannose–BSA (Yang et al., 1997), and binding is mediated by MBP. Consistent with these findings, we observed that the initial binding of *Acanthamoeba* to both cell types was mediated by MBP. Moreover, we have recently shown that *Acanthamoeba* isolates exhibiting higher levels of MBP expression showed increased cytotoxicity to host cells (Alsam et al., 2003), indicating that MBP is a potential marker for the differentiation of pathogenic *Acanthamoeba*. As indicated above, *Acanthamoeba*–mediated host cell cytotoxicity did not exhibit tissue specificity. This may be due to the longer incubations (24 h) required for cytotoxicity assays. Further assays of cell death that require shorter incubations may help to determine whether *Acanthamoeba*–mediated host cell death is genotype dependent or requires tissue specificity. Overall, these data suggest that the initial binding of pathogenic *Acanthamoeba* is mediated by MBP, but isolates expressing MBP undoubtedly possess other virulence factors that determine their ability to produce GAE, keratitis or both. Further studies are in progress to address these issues.

We next determined the effects of *Acanthamoeba* on the host cell cycle, both at the transcriptional and the protein level. A cell-cycle-specific gene array was employed. It was interesting to note that we observed a greater than 2.5-fold increase in GADD45 gene expression. GADD45 protein is known to inhibit CDK1 by physically dissociating the CDK1–cyclin B complex, eliminating CDK1 activity and resulting in G2-to-M checkpoint arrest (Zhan et al., 1999). In the normal cell cycle, CDK1 is phosphorylated when bound to cyclin B, which subsequently leads to phosphorylation of structural proteins in the nucleus including nucleolin, nuclear lamins and vimentins, leading to the initiation of mitosis and cellular division. However, our data suggested that *Acanthamoeba* induced increased levels of GADD45 gene expression, resulting in CDK1 inhibition leading to G2-to-M phase checkpoint arrest. Another interesting finding was the significantly increased level of RBL2 (p130RB2) gene expression. RBL2 is a member of the pRb family, which are critical regulators of G1-to-S phase transition in the cell cycle. Members of the pRb family include pRb, p130 and p107, which associate with E2F transcriptions factors, regulators of the cell cycle (Harbour & Dean, 2000; Dyson, 1998; Stevaux & Dyson, 2002). So far, six E2F proteins have been identified, divided into three categories. E2F1–E2F3 bind exclusively to pRb and are known to be transcriptional activators required to induce S-phase entry. E2F4 binds with high affinity to p107 and p130, and E2F5 associates with p130. E2F6 does not bind to any pRb family proteins but associates with Polycomb proteins. However, E2F4–E2F6 are known to act as transcriptional repressors. Moreover, p130–E2F complexes are found in quiescent or differentiated cells, while p107–E2F complexes are found in S-phase cells. Our data demonstrated that *Acanthamoeba* induces increased levels of p130 gene expression, clearly indicating that host cells undergo G1-to-S phase checkpoint arrest in response to *Acanthamoeba*. It was interesting to note that we did not observe any changes in the levels of pRb gene expression. This was an interesting finding, as it is well documented that the role of pRb is crucial in cell-cycle regulation. One possible explanation for our findings is that structural modifications of pRb occurred, rather than changes in the amounts of pRb in the cells. Indeed, pRb binds directly to the transactivation domain of E2F and blocks the ability of E2F to activate transcription of genes (G1 arrest), which is required for G1-to-S phase progression. However, upon stimulation to enter S phase, pRb becomes phosphorylated by CDKs at Ser249/252, Thr373, Ser780, Ser795 and Ser807/811 residues. This leads to pRb dissociation from the E2F transcription factors, allowing E2F to relocate to the nucleus and act as a transcriptional activator. To determine pRb modifications at the protein level, we used three antibodies generated against phospho-pRb Ser780, Ser795 and Ser807/811, and a control antibody generated against total pRb. We observed that *Acanthamoeba* induced pRb dephosphorylation in host cells, a marker of G1-to-S phase checkpoint arrest. Although we observed pRb dephosphorylation in both HBMEC and HCEC, HCEC required longer incubations with *Acanthamoeba* to exhibit similar effects. This delayed response was observed with both the T1 and T4 isolates. This could be due to the cells being of different origins (corneal epithelial cells versus brain microvascular endothelial cells) and/or the fact that HCEC are immortalized cells while HBMEC are primary in nature. Further studies are in progress to address these issues.
issues. Overall, we observed that Acanthamoeba produced pRb dephosphorylation and induced G1-to-S phase checkpoint arrest.

It is important to emphasize that cell-cycle arrest is the outcome of a complex signalling network that requires a fine balance between growth-stimulating and growth-inhibition pathways and is not as straightforward as discussed here. For example, some genes such as MCM7 and CDC27 exhibited gene expression in accordance with cell-cycle progression rather than cell-cycle arrest. However, the majority of genes analysed exhibited gene expression in compliance with cell-cycle arrest, which contributes to the overwhelming signalling pathways that result in the cellular decision to undergo cell-cycle arrest.

In conclusion, we have shown for the first time that Acanthamoeba induces differential expression of various genes crucial for G1-to-S transition and G2-to-M transitions with the collective response of host cell-cycle inhibition. These results were confirmed at the protein level by showing that Acanthamoeba abolished pRb phosphorylation in host cells and induced G1-to-S phase checkpoint arrest. Further understanding of the mechanisms associated with Acanthamoeba-host cell interactions will undoubtedly help to develop novel targets to treat Acanthamoeba infections.

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