Balamuthia mandrillaris interactions with human brain microvascular endothelial cells *in vitro*

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Balamuthia amoebic encephalitis (BAE) is a serious human disease almost always leading to death. An important step in BAE is amoebae invasion of the bloodstream, followed by their haematogenous spread. *Balamuthia mandrillaris* entry into the central nervous system most likely occurs at the blood–brain barrier sites. Using human brain microvascular endothelial cells (HBMECs), which constitute the blood–brain barrier, this study determined (i) the ability of *B. mandrillaris* to bind to HBMECs and (ii) the associated molecular mechanisms. Adhesion assays revealed that *B. mandrillaris* exhibited greater than 90% binding to HBMECs *in vitro*. To determine whether recognition of carbohydrate moieties on the surface of the HBMECs plays a role in *B. mandrillaris* adherence to the target cells, adhesion assays were performed in the presence of the saccharides mannose, galactose, xylose, glucose and fucose. It was observed that adherence of *B. mandrillaris* was significantly reduced by galactose, whilst the other saccharides had no effect. Acetone fixation of amoebae, but not of HBMECs, abolished adhesion, suggesting that *B. mandrillaris* adhesin(s) bind to galactose-containing glycoproteins of HBMECs. *B. mandrillaris* also bound to microtitre wells coated with galactose–BSA. By affinity chromatography using a galactose–Sephacrose column, a galactose-binding protein (GBP) was isolated from detergent extracts of unlabelled amoebae. The isolation of a GBP from cell-surface-biotin-labelled amoebae suggested its membrane association. One-dimensional SDS-PAGE confirmed the proteinaceous nature of the GBP and determined its molecular mass as approximately 100 kDa. This is the first report suggesting the role of a GBP in *B. mandrillaris* interactions with HBMECs.

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INTRODUCTION

Balamuthia mandrillaris is an emerging protozoan pathogen that can cause life-threatening infections involving the central nervous system (Schuster & Visvesvara, 2004; Visvesvara *et al.*, 1990, 1993). *Balamuthia* amoebic encephalitis (BAE) is characterized by headache, fever, characteristic skin lesions, stiff neck, nausea, vomiting, an acute confused state, cranial nerve palsies, seizures and finally death (Jayasekera *et al.*, 2004; Schuster & Visvesvara, 2004). Although there have been advances in the diagnosis of BAE (Boonton *et al.*, 2003a, b; Huang *et al.*, 1999; Qvarnstrom *et al.*, 2006; Tavares *et al.*, 2006; Yagi *et al.*, 2005), the pathogenesis and pathophysiology of this disease remain incompletely understood. Perhaps the most distressing aspect is the limited availability of effective

and/or recommended treatments against BAE. The infectious process involves amoebae invasion of the intravascular space through either the respiratory tract or skin lesions, followed by haematogenous spread. *B. mandrillaris* entry into the central nervous system most likely occurs at sites of the blood–brain barrier (Martinez *et al.*, 2001; Schuster & Visvesvara, 2004). However, the precise mechanisms by which *B. mandrillaris* transmigrates the blood–brain barrier are unclear. Using human brain microvascular endothelial cells (HBMECs), which constitute the blood–brain barrier, we have recently shown that *B. mandrillaris* produces severe host-cell cytotoxicity (Jayasekera *et al.*, 2004; Matin *et al.*, 2006, 2007). However, *B. mandrillaris*-mediated HBMEC cytotoxicity is a delayed event and requires parasite incubation with the host cells for over 24 h. Here, we studied *B. mandrillaris* primary attachment to HBMECs and attempted to identify the associated molecular mechanisms. The carbohydrate moieties of the host-cell plasma membrane are major

Abbreviations: BAE, *Balamuthia* amoebic encephalitis; GBP, galactose-binding protein; HBMEC, human brain microvascular endothelial cell; LDH, lactate dehydrogenase.

adhesion determinants for a number of pathogens including bacterial, fungal and protozoan pathogens (Alsam *et al.*, 2003; Garate *et al.*, 2006; Hostetter, 1994; Karlsson, 1989). Of interest, recent studies have shown that adhesion of *B. mandrillaris* to the extracellular matrix protein laminin can be blocked using exogenous galactose (Rocha-Azevedo *et al.*, 2007). In the present study, we revealed that *B. mandrillaris* binds to HBMECs and demonstrated the presence of a galactose-binding protein (GBP) on the surface membrane of *B. mandrillaris*.

METHODS

Culture of *B. mandrillaris*. *B. mandrillaris* isolated from the brain of a mandrill baboon (ATCC 50209) was routinely cultured as described previously (Jayasekera *et al.*, 2004; Matin *et al.*, 2006). Briefly, *B. mandrillaris* was inoculated (10^6 parasites) on confluent host-cell monolayers grown in T-75 tissue culture flasks. The amoebae consumed HBMECs within 48 h and produced approximately 5×10^6 – 8×10^6 parasites ($>99\%$ in trophozoite form), which were used for subsequent experiments.

Cultures of HBMECs. Primary HBMECs were grown in RPMI 1640 with 20% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, penicillin (100 U ml^{-1}), streptomycin ($100 \text{ } \mu\text{g ml}^{-1}$), non-essential amino acids and vitamins as described previously (Alsam *et al.*, 2003; Stins *et al.*, 1997).

Adhesion assays. To determine the effects of saccharides on *B. mandrillaris* binding to HBMECs, adhesion assays were performed in the presence of various saccharides as described previously (Sissons *et al.*, 2006). Briefly, HBMECs were grown in 24-well plates by inoculating 5×10^5 cells per well and the plates were incubated at 37°C in a 5% CO_2 incubator. Once confluent, HBMEC monolayers were incubated with *B. mandrillaris* (5×10^5 amoebae per well) in serum-free medium (RPMI 1640 containing 2 mM glutamine, 1 mM pyruvate and non-essential amino acids) and the plates were incubated at 37°C in a 5% CO_2 incubator for 1 h. After incubation, unbound amoebae were counted using a haemocytometer and the number of bound amoebae was calculated as follows: % unbound amoebae = (no. of unbound amoebae/total number of amoebae) $\times 100$. The number of bound amoebae was deduced as follows: % bound amoebae = $100 - \%$ unbound amoebae. To test the effect of saccharides, 2×10^5 amoebae were incubated with various saccharides [α -D-mannopyranoside (α -mannose), xylose, glucose, fucose and β -galactose at final concentrations of 10, 50 and 100 mM] in 100 μl RPMI 1640 for 30 min prior to the adhesion assay. In some adhesion assays, *B. mandrillaris* or HBMECs were fixed with 100% ice-cold acetone prior to adhesion assays (Jayasekera *et al.*, 2004). In addition, 24-well plates were coated with galactose-BSA and mannose-BSA ($1 \text{ } \mu\text{g ml}^{-1}$ final concn) in coating buffer (35 mM NaHCO_3 , 15 mM Na_2CO_3) and incubated for 24 h at 4°C . After incubation, wells were washed with PBS and inoculated with *B. mandrillaris* (5×10^5 amoebae per well). Plates were incubated at 37°C for 1 h to allow parasite binding. Finally, wells were washed with PBS and observed under a microscope.

Cytotoxicity assays. To determine the effects of saccharides on *B. mandrillaris*-mediated HBMEC death, cytotoxicity assays were performed as described previously (Kiderlen *et al.*, 2006; Sissons *et al.*, 2005). Briefly, *B. mandrillaris* was incubated with HBMECs as described for the adhesion assays and the plates were incubated for up to 24 h. At the end of this incubation period, supernatants were collected and the cytotoxicity was determined by measuring lactate

dehydrogenase (LDH) release using a cytotoxicity detection kit (Roche Applied Science). Briefly, conditioned medium of co-cultures of *B. mandrillaris* and HBMECs was collected and the percentage of LDH was detected as follows: % cytotoxicity = (test value–control value)/(total LDH release–control value) $\times 100$. Control values were obtained from HBMECs incubated alone and total LDH release was measured from HBMECs treated with 5% Triton X-100 for 1 h at 37°C .

Isolation of the GBP from *B. mandrillaris* trophozoites. To isolate the GBP of *B. mandrillaris*, cell pellets (10^8 trophozoites) were washed three times in 20 ml wash buffer [50 mM Tris/HCl (pH 7.2), 150 mM NaCl, 50 mM CaCl_2 , 1 mM PMSF] as described previously (Yang *et al.*, 1997). The cell pellets were then resuspended in lysis buffer (wash buffer plus 0.5% CHAPS and 2 mM β -mercaptoethanol) and disrupted with a sonifier (model 250; Branson Ultrasonics) using a tapered microtip at an output setting of 20 W. The amoebae extracts were clarified by centrifugation at 50 000 g for 1 h at 4°C , followed by chromatography on a galactose-Sepharose column. Unbound proteins were removed by washing the column with wash buffer, and bound proteins were eluted in 0.5 ml fractions with wash buffer containing 150 mM galactose. The bound fraction was dialysed, lyophilized and analysed by SDS-PAGE and the protein components in the gel were visualized with either silver or Coomassie blue staining as described previously (Yang *et al.*, 1997).

The functional role of the GBP was determined in adhesion assays as described above. Briefly, the GBP was pre-incubated with HBMECs for 30 min, followed by the addition of *B. mandrillaris*.

To determine whether the GBP was expressed on cell-surface membranes, intact amoebae were incubated with a surface-impermeable reagent [0.5 mg EZ-Link Sulfo-NHS-Biotin (Pierce) in 1 ml PBS for 30 min at room temperature] (Yang *et al.*, 1997) that covalently attaches biotin to proteins on the outer surface of intact cells, followed by lysis of amoebae and extraction of the GBP as described above. The biotinylated proteins eluted from the column were electrophoresed and blotted onto nitrocellulose membranes. The membranes were blocked using blocking buffer [25 mM Tris/HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20] containing 4% skimmed milk for 60 min at 22°C . Next, the biotinylated proteins were visualized using a Vectastain ABC-Amp kit (Vector Laboratories) for 1 h. Finally, blots were washed and developed using an enhanced chemiluminescence kit (Pharmacia Biosciences).

RESULTS AND DISCUSSION

B. mandrillaris exhibits binding to HBMECs in a galactose-inhibitable manner

To determine whether the carbohydrate moieties on the surface of the HBMECs are involved in *B. mandrillaris* interactions, adhesion assays were performed in the presence of the saccharides α -mannose, xylose, glucose, fucose and β -galactose (final concentrations of 10, 50 and 100 mM). The results revealed that *B. mandrillaris* exhibited greater than 90% binding to HBMECs and, of the various saccharides tested, only exogenous galactose inhibited binding of amoebae to HBMECs in a concentration-dependent manner (Fig. 1). Of interest, *B. mandrillaris* bound to microtitre wells coated with neoglycoprotein galactose-BSA but not to mannose-BSA-coated wells (data not shown). To confirm further that, at least primarily, *B. mandrillaris* recognized carbohydrate moieties on the

Fig. 1. *B. mandrillaris* exhibits binding to HBMECs in a galactose-inhibitable manner. To determine whether carbohydrate moieties on the surface of HBMECs are involved in *B. mandrillaris* interactions, adhesion assays were performed in the presence of various saccharides as described in Methods. Briefly, confluent monolayers of HBMECs were incubated with *B. mandrillaris* (5×10^5 amoebae per well). Plates were incubated at 37 °C in a 5% CO₂ incubator for 1 h and bound amoebae were enumerated. To test the effect of saccharides, assays were performed in the presence of various saccharides [α -D-mannopyranoside (α -mannose), xylose, glucose, fucose and β -galactose at final concentrations of 10, 50 and 100 mM]. Note that *B. mandrillaris* exhibited greater than 90% binding to HBMECs and, of the various saccharides, only exogenous galactose inhibited binding of amoebae to HBMECs in a concentration-dependent manner. The results represent the mean of three independent experiments performed in duplicate.

surface of the HBMECs, adhesion assays were performed using acetone-fixed amoebae or HBMECs. Acetone fixation denatures proteins but does not affect saccharides. As shown in Fig. 2, fixation of amoebae, but not of HBMECs, abolished *B. mandrillaris* adhesion to the host cells, suggesting that the molecular binding partners were protein(s) of the parasite and carbohydrate chains of plasma membrane glycoconjugates of the host cells.

Fig. 2. To determine whether *B. mandrillaris* recognizes carbohydrate moieties on the surface of HBMECs, adhesion assays were performed using acetone-fixed amoebae or HBMECs as described in Methods. Briefly, confluent monolayers of HBMECs or amoebae were fixed with 100% ice-cold acetone and adhesion assays were performed as described in Fig. 1. Acetone fixation of amoebae, but not of HBMECs, abolished *B. mandrillaris* adhesion to the host cells, suggesting that the molecular binding partners are protein(s) of the parasite and carbohydrate chains of plasma membrane glycoconjugates of the host cells. The results represent the mean of three independent experiments performed in duplicate.

Exogenous galactose has no effect on *B. mandrillaris*-mediated HBMEC cytotoxicity

Previous studies have shown that *Entamoeba histolytica* adhesion to host cells can be blocked using exogenous galactose/*N*-acetylgalactosamine, which also inhibits parasite-mediated host-cell cytotoxicity (reviewed by Pillai & Kain, 2000; Ravdin, 1989). These findings suggest that *E. histolytica*-mediated host-cell death is a contact-dependent event. As exogenous galactose inhibited *B. mandrillaris* adhesion to the host cells, we next determined the effects of galactose on *B. mandrillaris*-mediated HBMEC cytotoxicity. Our findings revealed that exogenous galactose had no significant effect on *B. mandrillaris*-mediated HBMEC cytotoxicity. In the absence of sugars, *B. mandrillaris* produced $73 \pm 7\%$ HBMEC cytotoxicity, whilst the presence of exogenous sugars had no significant effect: amoebae produced $78.5 \pm 0.5\%$ HBMEC cytotoxicity in the presence of 100 mM galactose, $76 \pm 8.4\%$ with 100 mM mannose, $78.5 \pm 9.1\%$ with 100 mM fucose, $81.5 \pm 9.1\%$ with 100 mM glucose and $74.5 \pm 6.3\%$ with 100 mM xylose.

B. mandrillaris expresses a GBP

To determine whether *B. mandrillaris* expressed a GBP, cell lysates were analysed by chromatography on a galactose-Sepharose column. Proteins that specifically bound to the affinity column were eluted with 150 mM galactose and analysed by SDS-PAGE. We observed a major component in the bound fraction with an approximate molecular mass of 100 kDa (Fig. 3a), suggesting that *B. mandrillaris* expresses a GBP (Fig. 3a). Next, to determine whether the GBP was membrane-associated, cell-surface proteins of intact *B. mandrillaris* were biotinylated, followed by

isolation of the GBP on a galactose–Sephacryl column and blotting using a Vectastain ABC–AmP kit as described in Methods. We observed a biotin-labelled component of the same molecular mass (approx. 100 kDa) as that found in the bound fraction of the unlabelled amoebae lysates (Fig. 3b). We also found another protein with an approximate molecular mass of 70 kDa. Overall, these findings suggested that the GBP is expressed on the cell-surface membranes of *B. mandrillaris*.

The GBP inhibits *B. mandrillaris* binding to and cytotoxicity of HBMECs

To determine the functional role of the GBP, adhesion assays were performed by pre-incubating HBMECs with the GBP. Our finding revealed that the GBP inhibited greater than 50 % of *B. mandrillaris* adhesion to HBMECs (Fig. 4a) ($P < 0.05$, using a paired *t*-test with one-tailed distribution), clearly indicating that the GBP expressed on the surface of parasites plays an important role in *B.*

mandrillaris adhesion to HBMECs. In contrast, the unbound fraction had no significant effect on binding of amoebae to HBMECs (Fig. 4a).

Next, to determine the effects of the GBP on *B. mandrillaris*-mediated HBMEC cytotoxicity, cytotoxicity assays were performed in the presence of the GBP. As shown in Fig. 4(b), the results revealed that the GBP inhibited *B. mandrillaris*-mediated HBMEC cytotoxicity ($P < 0.05$ using a paired *t*-test with one-tailed distribution). Overall, these results suggested that the GBP may play important roles in *B. mandrillaris* interactions with HBMECs.

Fig. 3. *B. mandrillaris* expresses a GBP. (a) To determine whether *B. mandrillaris* expresses a GBP, cell lysates were analysed by chromatography on a galactose–Sephacryl column followed by SDS-PAGE as described in Methods. Briefly, *B. mandrillaris* cell pellets (10^8 trophozoites) were lysed and disrupted with a sonifier. The amoebae extracts were clarified by centrifugation at 500 000 *g* for 1 h at 4 °C and analysed on a galactose–Sephacryl column. Unbound proteins were removed and bound proteins were eluted with 150 mM galactose. The bound fraction was dialysed, lyophilized and analysed by SDS-PAGE. A major component was detected in the bound fraction with an approximate molecular mass of 100 kDa. (b) To determine whether GBP is membrane-associated, cell-surface proteins of intact *B. mandrillaris* were biotinylated as described in Methods, followed by extraction of the GBP as above. A biotin-labelled component of the same molecular mass (approx. 100 kDa) as that found in the bound fraction of the unlabelled amoebae lysates was observed. The results are representative of three independent experiments.

Fig. 4. GBP inhibits *B. mandrillaris* binding to and cytotoxicity towards HBMECs. (a) To determine the functional role of the GBP, adhesion assays were performed by pre-incubating HBMECs with the GBP as described in Methods. The GBP inhibited more than 50 % of *B. mandrillaris* adhesion to HBMECs ($P < 0.05$, using a paired *t*-test with one-tailed distribution). In contrast, the unbound fraction had no significant effect on binding of amoebae to HBMECs. (b) To determine the effects of the GBP on *B. mandrillaris*-mediated HBMEC death, cytotoxicity assays were performed in the presence of the GBP as described in Methods. Briefly, HBMECs were incubated with the GBP for 30 min followed by inoculation of *B. mandrillaris*. Plates were then incubated for up to 24 h. At the end of the incubation period, supernatants of co-cultures of *B. mandrillaris* and HBMECs were collected and the percentage of LDH release was determined. The results showed that the GBP inhibited *B. mandrillaris*-mediated HBMEC cytotoxicity.

One of the key steps in the pathogenesis of BAE is *B. mandrillaris* invasion of the central nervous system, which probably occurs at sites of the blood–brain barrier. Using HBMECs, which constitute the blood–brain barrier, we have recently shown that *B. mandrillaris* produces HBMEC cytotoxicity, which may lead to blood–brain barrier perturbations (Matin *et al.*, 2006, 2007). However, the underlying molecular mechanisms associated with amoebae traversal of the blood–brain barrier leading to pathological features remain unclear. Although the successful traversal of *B. mandrillaris* across the blood–brain barrier may require multiple events, we hypothesize that adhesion is a primary step in amoebae transmigration of the HBMECs. In this study, we demonstrated that *B. mandrillaris* binds to HBMECs in a galactose-inhibitable manner and identified a GBP expressed on the surface of *B. mandrillaris*. The inhibition of *B. mandrillaris* adhesion to HBMECs was specific to galactose, as other saccharides had no effect on amoebae binding. To confirm further that the galactose-containing glycoproteins on HBMECs serve as attachment sites for *B. mandrillaris*, adhesion assays were performed by fixing either amoebae or HBMECs. We observed that acetone fixation of amoebae abolished *B. mandrillaris* binding to HBMECs, whilst amoebae bound to acetone-fixed HBMECs in a galactose-inhibitable manner, suggesting that the parasite binds to galactose residues on HBMECs. The presence of a GBP in *B. mandrillaris* has recently been suggested by Rocha-Azevedo *et al.* (2007), who demonstrated that *B. mandrillaris* binds to laminin and that these interactions can be inhibited using exogenous galactose. Our results support these findings and have identified the expression of a GBP on the surface membranes of *B. mandrillaris*. However, given the complexity of host–parasite interactions, it is tempting to speculate that the aforementioned interactions only provide initial attachment, which is likely to be followed by closer associations with a more intimate contact of *B. mandrillaris* to HBMECs involving the GBP as well as other adhesin(s). Such binding is probably necessary to withstand blood flow, as well as for subsequent crossing of the blood–brain barrier. Further studies are needed to validate the concept of other determinants, in addition to GBP, and their roles in *B. mandrillaris*–HBMEC interactions.

Using chromatography of extracts of *B. mandrillaris* on a galactose–Sepharose column, we isolated a GBP with an approximate molecular mass of 100 kDa. Previous studies have identified a galactose/*N*-acetylgalactosamine lectin in *Entamoeba*. The isolation and characterization of this amoebic lectin revealed that it was a 260 kDa heterodimer, composed of a 170 kDa heavy subunit and a 31/35 kDa light subunit that is anchored by glycosylphosphatidylinositol to the surface membrane bilayer (reviewed by Pillai & Kain, 2000; Ravdin, 1989). Interestingly, Ravdin & Guerrant (1981) showed that the *E. histolytica* lectin is important in adherence and contact-dependent cytolysis of human cells. This was shown using exogenous

galactose/*N*-acetylgalactosamine, which blocked *E. histolytica* binding to and killing of target cells, suggesting that *E. histolytica*-mediated human cell cytotoxicity is a contact-dependent process. In contrast, our findings revealed that, although exogenous galactose blocked *B. mandrillaris* binding to HBMECs, it had no effect on *B. mandrillaris*-mediated HBMEC death. We are cautious in interpreting these findings as our cytotoxicity assays were performed by incubating *B. mandrillaris* with HBMECs over longer periods of time, i.e. 24 h. Of interest, the GBP inhibited *B. mandrillaris*-mediated HBMEC cytotoxicity, suggesting the involvement of diverse mechanisms. Overall, these findings suggest that *B. mandrillaris*-mediated HBMEC cytotoxicity may involve both contact-dependent and -independent mechanisms. Future studies are in progress to address these issues.

In conclusion, we have shown for the first time that *B. mandrillaris* primarily binds to galactose residues of the plasma membrane glycoconjugates of the host cells and identified a GBP expressed on the cell surface of these parasites. Further studies will characterize the GBP and determine its potential role in *B. mandrillaris* pathogenesis, which may identify novel targets for the rational development of therapeutic interventions and/or design of preventative strategies. This is not a novel concept. For example, *Acanthamoeba*, a close relative of *Balamuthia*, is a causative agent of blinding keratitis. Recent studies have shown that *Acanthamoeba* binds to corneal epithelial cells using its mannose-binding protein (Garate *et al.*, 2004; Morton *et al.*, 1991; Yang *et al.*, 1997). Interestingly, oral immunization with recombinant mannose-binding protein protected against *Acanthamoeba* keratitis *in vivo*. Similar strategies may be developed against BAE and the identification of the GBP should lay a foundation for future studies.

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REFERENCES

- Alsam, S., Kim, K. S., Stins, M., Rivas, A. O., Sissons, J. & Khan, N. A. (2003). *Acanthamoeba* interactions with human brain microvascular endothelial cells. *Microb Pathog* **35**, 235–241.
- Booton, G. C., Schuster, F. L., Carmichael, J. R., Fuerst, P. A. & Byers, T. J. (2003a). *Balamuthia mandrillaris*: identification of clinical and environmental isolates using genus-specific PCR. *J Eukaryot Microbiol* **50**, 508–509.
- Booton, G. C., Carmichael, J. R., Visvesvara, G. S., Byers, T. J. & Fuerst, P. A. (2003b). Identification of *Balamuthia mandrillaris* by PCR assay using the mitochondrial 16S rRNA gene as a target. *J Clin Microbiol* **41**, 453–455.
- Garate, M., Cao, Z., Bateman, E. & Panjwani, N. (2004). Cloning and characterization of a novel mannose-binding protein of *Acanthamoeba*. *J Biol Chem* **279**, 29849–29856.

- Garate, M., Marchant, J., Cubillos, I., Cao, Z., Khan, N. A. & Panjwani, N. (2006). *In vitro* pathogenicity of *Acanthamoeba* is associated with the expression of the mannose-binding protein. *Invest Ophthalmol Vis Sci* 47, 1056–1062.
- Hostetter, M. K. (1994). Adhesins and ligands involved in the interaction of *Candida* spp. with epithelial and endothelial surfaces. *Clin Microbiol Rev* 7, 29–42.
- Huang, Z. H., Ferrante, A. & Carter, R. F. (1999). Serum antibodies to *Balamuthia mandrillaris*, a free-living amoeba recently demonstrated to cause granulomatous amoebic encephalitis. *J Infect Dis* 179, 1305–1308.
- Jayasekera, S., Sissons, J., Tucker, J., Rogers, C., Nolder, D., Warhurst, D., Alsam, S., White, J. M. L., Higgins, E. M. & Khan, N. A. (2004). Post mortem culture of *Balamuthia mandrillaris* from the brain and cerebrospinal fluid of a case of granulomatous amoebic meningoencephalitis, using human brain microvascular endothelial cells. *J Med Microbiol* 53, 1007–1012.
- Karlsson, K. A. (1989). Animal glycosphingolipids as membrane attachment sites for bacteria. *Annu Rev Biochem* 58, 309–350.
- Kiderlen, A. F., Tata, P. S., Ozel, M., Laube, U., Radam, E. & Schafer, H. (2006). Cytopathogenicity of *Balamuthia mandrillaris*, an opportunistic causative agent of granulomatous amoebic encephalitis. *J Eukaryot Microbiol* 53, 456–463.
- Martinez, A. J., Schuster, F. L. & Visvesvara, G. S. (2001). *Balamuthia mandrillaris*: its pathogenic potential. *J Eukaryot Microbiol* (Suppl.), 6S–9S.
- Matin, A., Stins, M., Kim, K. S. & Khan, N. A. (2006). *Balamuthia mandrillaris* exhibit metalloprotease activities. *FEMS Immunol Med Microbiol* 47, 83–91.
- Matin, A., Jeong, S. R., Stins, M. & Khan, N. A. (2007). Effects of human serum on *Balamuthia mandrillaris* interactions with the human brain microvascular endothelial cells. *J Med Microbiol* 56, 30–35.
- Morton, L. D., McLaughlin, G. L. & Whiteley, H. E. (1991). Effect of temperature, amoebic strain and carbohydrates on *Acanthamoeba* adherence to corneal epithelium *in vitro*. *Infect Immun* 59, 3819–3822.
- Pillai, D. R. & Kain, K. C. (2000). Recent developments in amoebiasis: the Gal/GalNAc lectins of *Entamoeba histolytica* and *Entamoeba dispar*. *Microbes Infect* 2, 1775–1783.
- Qvarnstrom, Y., Visvesvara, G. S., Sriram, R. & da Silva, A. J. (2006). Multiplex real-time PCR assay for simultaneous detection of *Acanthamoeba* spp., *Balamuthia mandrillaris*, and *Naegleria fowleri*. *J Clin Microbiol* 44, 3589–3595.
- Ravdin, J. I. (1989). *Entamoeba histolytica*: from adherence to enteropathy. *J Infect Dis* 159, 420–429.
- Ravdin, J. I. & Guerrant, R. L. (1981). Role of adherence in cytopathogenic mechanisms of *Entamoeba histolytica*. Study with mammalian tissue culture cells and human erythrocytes. *J Clin Invest* 68, 1305–1313.
- Rocha-Azevedo, B., Jamerson, M., Cabral, G. A., Silva-Filho, F. C. & Marciano-Cabral, F. (2007). The interaction between the amoeba *Balamuthia mandrillaris* and extracellular matrix glycoproteins *in vitro*. *Parasitology* 134, 51–58.
- Schuster, F. L. & Visvesvara, G. S. (2004). Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Int J Parasitol* 34, 1001–1027.
- Sissons, J., Kim, K. S., Stins, M., Jayasekera, S., Alsam, S. & Khan, N. A. (2005). *Acanthamoeba castellanii* induces host cell death via a phosphatidylinositol 3-kinase-dependent mechanism. *Infect Immun* 73, 2704–2708.
- Sissons, J., Alsam, S., Stins, M., Rivas, A. O., Morales, J. L., Faull, J. & Khan, N. A. (2006). Use of *in vitro* assays to determine effects of human serum on biological characteristics of *Acanthamoeba castellanii*. *J Clin Microbiol* 44, 2595–2600.
- Stins, M. F., Gilles, F. & Kim, K. S. (1997). Selective expression of adhesion molecules on human brain microvascular endothelial cells. *J Neuroimmunol* 76, 81–90.
- Tavares, M., Correia da Costa, J. M., Carpenter, S. S., Santos, L. A., Afonso, C., Aguiar, A., Pereira, J., Cardoso, A. I., Schuster, F. L. & other authors (2006). Diagnosis of first case of *Balamuthia* amoebic encephalitis in Portugal by immunofluorescence and PCR. *J Clin Microbiol* 44, 2660–2663.
- Visvesvara, G. S., Martinez, A. J., Schuster, F. L., Leitch, G. J., Wallace, S. V., Sawyer, T. K. & Anderson, M. (1990). Leptomyxid amoeba, a new agent of amoebic meningoencephalitis in humans and animals. *J Clin Microbiol* 28, 2750–2756.
- Visvesvara, G. S., Schuster, F. L. & Martinez, A. J. (1993). *Balamuthia mandrillaris*, N. G., N. Sp., agent of amoebic meningoencephalitis in humans and other animals. *J Eukaryot Microbiol* 40, 504–514.
- Yagi, S., Booton, G. C., Visvesvara, G. S. & Schuster, F. L. (2005). Detection of *Balamuthia* mitochondrial 16S rRNA gene DNA in clinical specimens by PCR. *J Clin Microbiol* 43, 3192–3197.
- Yang, Z., Cao, Z. & Panjwani, N. (1997). Pathogenesis of *Acanthamoeba* keratitis: carbohydrate-mediated host–parasite interactions. *Infect Immun* 65, 439–445.