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–Sepharose 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.

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 (Boothon 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 (Aksam 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 (10⁶ parasites) on confluent host-cell monolayers grown in T-75 tissue culture flasks. The amoebae consumed HBMECs within 48 h and produced approximately 5×10⁵–8×10⁶ 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 µg ml⁻¹), non-essential amino acids and vitamins as described previously (Aksam 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 5×10⁵ cells per well and the plates were incubated at 37 °C in a 5% CO₂ incubator. Once confluent, HBMEC monolayers were incubated with *B. mandrillaris* (5×10⁵ 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% CO₂ 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) × 100. The number of bound amoebae was deduced as follows: % bound amoebae=100-% unbound amoebae. To test the effect of saccharides, 2×10⁵ amoebae were incubated with various saccharides [α-galactoside (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 µg ml⁻¹ final concn) in coating buffer (35 mM NaHCO₃, 15 mM Na₂CO₃) and incubated for 24 h at 4 °C. After incubation, wells were washed with PBS and inoculated with *B. mandrillaris* (5×10⁵ 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) ×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 (10⁸ trophozoites) were washed three times in 20 ml wash buffer [50 mM Tris/HCl (pH 7.2), 150 mM NaCl, 50 mM CaCl₂, 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, lyophilised 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
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.

**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 ± 7 % HBMEC cytotoxicity, whilst the presence of exogenous sugars had no significant effect: amoebae produced 78.5 ± 0.5 % HBMEC cytotoxicity in the presence of 100 mM galactose, 76 ± 8.4 % with 100 mM mannose, 78.5 ± 9.1 % with 100 mM fucose, 81.5 ± 9.1 % with 100 mM glucose and 74.5 ± 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–Sepharose 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–Sepharose column followed by SDS-PAGE as described in Methods. Briefly, *B. mandrillaris* cell pellets (10⁸ 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–Sepharose 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 adhesion(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 & Bain, 2000; Radvin, 1989). Interestingly, Radvin & Guarrant (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**


