Effects of human serum on *Balamuthia mandrillaris* interactions with human brain microvascular endothelial cells

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Balamuthia mandrillaris is a free-living amoeba and a causative agent of fatal granulomatous encephalitis. In the transmission of *B. mandrillaris* into the central nervous system (CNS), haematogenous spread is thought to be the primary step, followed by blood–brain barrier penetration. The objectives of the present study were (i) to determine the effects of serum from healthy individuals on the viability of *B. mandrillaris*, and (ii) to determine the effects of serum on *B. mandrillaris*-mediated blood–brain barrier perturbations. It was determined that normal human serum exhibited limited amoebicidal effects, i.e. ~40 % of trophozoites were killed. The residual subpopulation, although viable, remained static over longer incubations. Using human brain microvascular endothelial cells (HBMEC), which form the blood–brain barrier, it was observed that *B. mandrillaris* exhibited binding (>80 %) and cytotoxicity (>70 %) to HBMEC. However, normal human serum exhibited more than 60 % inhibition of *B. mandrillaris* binding and cytotoxicity to HBMEC. ELISAs showed that both serum and saliva samples exhibit the presence of anti-*B. mandrillaris* antibodies. Western blots revealed that normal human serum reacted with several *B. mandrillaris* antigens with approximate molecular masses of 148, 115, 82, 67, 60, 56, 44, 42, 40 and 37 kDa. Overall, the results demonstrated that normal human serum has inhibitory effects on *B. mandrillaris* growth and viability, as well as on their binding and subsequent cytotoxicity to HBMEC. A complete understanding of *B. mandrillaris* pathogenesis is crucial to develop therapeutic interventions and/or to design preventative measures.

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INTRODUCTION

First discovered in 1986 by G. S. Visvesvara (Centers for Disease Control, USA), *Balamuthia mandrillaris* is an opportunistic protozoan pathogen that can cause fatal human infection involving the central nervous system (CNS) (Anzil *et al.*, 1991; Taratuto *et al.*, 1991; Visvesvara *et al.*, 1990, 1993). *Balamuthia* granulomatous encephalitis (BGE) is characterized by headache, fever, characteristic skin lesions, stiff neck, nausea, vomiting, acute confused state, with cerebral haemorrhagic necrotizing lesions detected by neuroimaging scans, cranial nerve palsies, seizures and finally death (Jayasekera *et al.*, 2004; reviewed by Schuster & Visvesvara, 2004). The granuloma is composed of CD4-, CD8-positive T lymphocytes, B lymphocytes, plasma cells, multinucleate giant cells and macrophages, but it may be

absent in severely immunocompromised patients (Schuster & Visvesvara, 2004). Although the predisposing factors in contracting BGE are not known, recent studies have shown that, unlike *Acanthamoeba*, *B. mandrillaris* can cause fatal infections in relatively immunocompetent people. This is shown by the findings that BGE can develop in patients with no history of syphilis, diabetes mellitus, malignancies, or fungal and mycobacterial infections, and who are negative for HIV-1 and HIV-2. The portal of entry into the CNS is thought to be the olfactory neuroepithelium (Kiederlan & Laube, 2004) or lower respiratory tract, followed by haematogenous spread. Skin lesions may provide direct entry into the bloodstream, bypassing the lower respiratory tract. In the case of haematogenous spread, amoebae entry into the CNS most likely occurs at the blood–brain barrier (Martinez *et al.*, 2001; Schuster & Visvesvara, 2004). Infection of the skin and lungs can last for months, but the involvement of the CNS results in death within days (Jayasekera *et al.*, 2004). Recent studies have shown that *B. mandrillaris* exhibits multifactorial properties to produce

Abbreviations: BGE, *Balamuthia* granulomatous encephalitis; CNS, central nervous system; HBMEC, human brain microvascular endothelial cells; LDH, lactate dehydrogenase; sIgA, secretory IgA.

damage of human brain microvascular endothelial cells (HBMEC), which form the blood–brain barrier (Jayasekera *et al.*, 2004, 2005; Matin *et al.*, 2006). However, the effects of normal human serum on *B. mandrillaris* interactions with HBMEC are not known and are the subject of the present study.

METHODS

HBMEC cultures. Primary HBMEC were obtained and cultured as previously described (Alsam *et al.*, 2003; Stins *et al.*, 1997). Briefly, HBMEC were routinely grown in 20% heat-inactivated fetal bovine serum, 2 mM glutamine, 1 mM pyruvate, 100 U penicillin ml⁻¹, 100 µg streptomycin ml⁻¹, non-essential amino acids, vitamins and RPMI 1640 (Invitrogen). For experiments, HBMEC were grown in 24-well plates by inoculating 5×10^5 cells in 1 ml into each well, and plates were incubated at 37 °C in a 5% CO₂ incubator. At this cell density, confluent monolayers were formed within 24 h, and these were used for subsequent experiments.

Cultures of *B. mandrillaris*. *B. mandrillaris* isolated from the brain of a mandrill baboon was obtained from the American Type Culture Collection (ATCC 50209). *B. mandrillaris* were routinely cultured on HBMEC as a food source, as previously described (Jayasekera *et al.*, 2004; Matin *et al.*, 2006). Briefly, *B. mandrillaris* were inoculated (10^6 parasites in 10 ml RPMI 1640) on HBMEC monolayers grown in T-75 tissue-culture flasks. *B. mandrillaris* consumed the HBMEC monolayer within 48 h, and produced approximately $5\text{--}8 \times 10^6$ parasites per 10 ml (>99% in trophozoite forms), which were used for subsequent experiments.

Adhesion assays. To determine whether normal human serum affects *B. mandrillaris* adhesion to HBMEC, adhesion assays were performed (Sissons *et al.*, 2005). Briefly, HBMEC were grown to confluency in 24-well plates. *B. mandrillaris* (2×10^5 amoebae) were pre-incubated with 20 and 100% human serum (obtained from healthy individuals by Harlan SeraLab, tested negative for hepatitis C virus, HIV-1, HIV-2 and hepatitis B surface antigen, and considered to be normal human serum). When purchased, each batch of serum was from one individual, and several batches were obtained. To test the involvement of complement pathways, assays were performed using heat-inactivated serum (56 °C for 30 min to inactivate complement components) as previously described (Toney & Marciano-Cabral, 1998). Next, amoebae plus serum were transferred to HBMEC monolayers in 24-well plates, and the plates were incubated at 37 °C in 5% CO₂ for 60 min. After this incubation, the unbound amoebae were counted using a haemocytometer, and the percentage of unbound amoebae was calculated from the following equation: (number unbound amoebae/total number amoebae) $\times$ 100. The percentage of bound amoebae was calculated from the following equation: 100 – percentage of unbound amoebae.

Cytotoxicity assays. Cytotoxicity assays were performed as previously described (Alsam *et al.*, 2003). Briefly, *B. mandrillaris* with or without human serum was incubated with HBMEC monolayers grown in 24-well plates, as described for adhesion assays. Plates were incubated at 37 °C in a 5% CO₂ incubator and periodically observed for cytopathic effects for up to 24 h. At the end of this incubation period, supernatants were collected and cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release (cytotoxicity detection kit; Roche Applied Science). Briefly, conditioned media of co-cultures of amoebae and HBMEC were collected, and the percentage cytotoxicity was calculated as follows: ((sample value – control value)/(total LDH release – control value)) $\times$ 100. Control values

were obtained from HBMEC incubated in RPMI alone. Total LDH release was determined from HBMEC treated with 1% Triton X-100 for 30 min at 37 °C.

Amoebicidal and amoebistatic assays. For amoebicidal assays, *B. mandrillaris* trophozoites were incubated with 20 and 100% normal human serum and heat-inactivated serum, as described above. The numbers of *B. mandrillaris* at various time intervals were determined using haemocytometer counting. The counts from *B. mandrillaris* incubated alone in the absence of serum were taken as 100%, and results are presented as a percentage relative to that value. For amoebistatic assays, *B. mandrillaris* trophozoites were added to HBMEC in the presence of normal human serum and heat-inactivated serum. At various time intervals, *B. mandrillaris* were washed from the surface of HBMEC monolayers and numbers were determined by haemocytometer counting. The counts from *B. mandrillaris* plus HBMEC in the absence of serum were taken as 100%, and results are presented as a percentage relative to that value.

Western blots. The presence of anti-*B. mandrillaris* IgG in human serum was determined by Western blots, as previously described (Khan *et al.*, 2002). Briefly, various numbers of *B. mandrillaris* were mixed (1:1) with sample buffer containing 10% β -mercaptoethanol and heated at 100 °C for 5 min. Samples were centrifuged at 10 000 g for 1 min and electrophoresed by 12.5% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. The membranes were blocked using blocking buffer (25 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing 4% skimmed milk for 60 min at 22 °C. After this incubation, the membranes were immunoblotted using normal human serum (1 ml) overnight at 4 °C. Next day, membranes were washed three times with PBS and incubated with an appropriate horseradish peroxidase-linked secondary antibody for 1 h. Finally, blots were washed and developed using an enhanced chemiluminescence kit (Amersham Biosciences).

ELISAs. The presence of anti-*B. mandrillaris* IgG in normal human serum and secretory IgA (sIgA) in mucosal secretions (saliva was obtained from healthy individuals) was determined using ELISAs, as previously described (Leher *et al.*, 1998). Briefly, *B. mandrillaris* were added to 96-well plates (2×10^5 amoebae per well). Subsequently, wells were air-dried, followed by the addition of ice-cold methanol and acetone (1:1) for 45 min. The wells were washed twice with PBS containing 0.05% Tween-20 to remove non-adherent amoebae, and blocked using 3% BSA for 1 h at 37 °C. The normal human serum and saliva samples were serially diluted from 1:1 to 1:50 000. *B. mandrillaris* were washed and 100 µl of test samples was added to the wells and incubated for 18 h at 4 °C. The following day, amoebae were washed five times with PBS plus Tween-20 and incubated with the respective secondary antibody. For *B. mandrillaris* incubated with saliva samples, wells were incubated with mouse anti-human IgA (Abcam) for 60 min and washed five times, as above. For *B. mandrillaris* incubated with serum, this step was omitted. Next, anti-mouse IgG antibody conjugated to horseradish peroxidase was added to all wells. The plates were incubated for 1 h at 37 °C. Finally, the wells were washed five times as above and 100 µl substrate solution (0.1% H₂O₂, 0.1% orthophenylenediamine in citrate buffer) was added. The reactions were allowed to develop for 15 min, and finally 100 µl 3% sulphuric acid was added to stop the reaction. The A₄₉₂ of each well was determined on a microplate reader (Anthos 2020, Jencons-PLS). The A₄₉₂ of *B. mandrillaris* incubated with secondary antibody in the absence of serum and saliva samples was taken as the background.

RESULTS AND DISCUSSION

Serum from healthy individuals inhibits *B. mandrillaris* adhesion to HBMEC

To determine the effects of normal human serum on *B. mandrillaris* binding to HBMEC, adhesion assays were performed. We observed that serum inhibited up to 50 % of amoeba binding to HBMEC monolayers (Fig. 1). Next, to determine the effects of heat inactivation on the inhibitory effects of serum, adhesion assays were performed using heat-inactivated serum. The results revealed that normal and heat-inactivated serum exhibited similar inhibitory effects on amoeba binding to HBMEC (Fig. 1).

Serum from healthy individuals inhibits *B. mandrillaris*-mediated HBMEC cytotoxicity

To determine the effects of normal human serum on *B. mandrillaris*-mediated HBMEC death, cytotoxicity assays were performed. In the absence of serum, *B. mandrillaris* produced severe HBMEC cell cytotoxicity (more than 70 %) within 24 h (Fig. 2). However, *B. mandrillaris*-mediated HBMEC cytotoxicity was inhibited in the presence of serum (Fig. 2). Again, heat-inactivation of serum did not abolish serum effects (Fig. 2). Overall, these data show that human serum partially inhibits *B. mandrillaris*-mediated HBMEC cytotoxicity.

Fig. 1. Serum from healthy individuals inhibits *B. mandrillaris* adhesion to HBMEC. HBMEC were grown to monolayers in 24-well plates and incubated with 2×10^5 *B. mandrillaris* for 1 h at 37 °C in the presence of 20 or 100 % normal human serum (NHS) or heat-inactivated serum (HI-NHS). Note that serum inhibited *B. mandrillaris* adhesion to HBMEC in a heat-inactivation-independent manner (* $P < 0.05$ using Student's *t* test with paired, two-tailed distribution). Results are representative of three independent experiments performed in triplicate; bars represent standard error.

Fig. 2. Serum from healthy individuals inhibits *B. mandrillaris*-mediated HBMEC cytotoxicity. *B. mandrillaris* were added to HBMEC monolayers in 24-well plates and incubated at 37 °C for up to 24 h. HBMEC without *B. mandrillaris* were used as controls. The percentage HBMEC cytotoxicity was determined by measuring LDH release, as described in Methods. Serum inhibits *B. mandrillaris*-mediated HBMEC cytotoxicity in a heat-inactivation-independent manner (* $P < 0.05$ using Student's *t* test with paired, two-tailed distribution). Results are representative of three independent experiments performed in triplicate; bars represent standard error.

Serum from healthy individuals induces an initial amoebicidal effect followed by amoebistatic activity

To determine whether the effects of serum on *B. mandrillaris* virulence properties are mediated via distinct molecular mechanisms or are simply secondary to the amoebistatic/amoebicidal properties of serum, assays were performed as described in Methods. Our findings revealed that 100 % serum exhibited partial amoebicidal effects, with an initial reduction in amoeba numbers (Fig. 3). However, a subpopulation of *B. mandrillaris* remained intact. In the presence of 100 % serum, cultures remained static over longer incubations (Fig. 4). However, in the presence of 20 % serum, a non-significant increase ($P > 0.05$) in the numbers of *B. mandrillaris* was observed. Furthermore, to determine whether this subpopulation of *B. mandrillaris* was viable, 50 µl culture suspension was inoculated onto HBMEC monolayers, and their viability was determined by Trypan blue exclusion assay. *B. mandrillaris* were negative for Trypan blue and appeared as viable trophozoites on HBMEC monolayers, suggesting that the effect of serum on the remaining population was amoebistatic (data not shown).

ELISA demonstrates the presence of *B. mandrillaris*-specific IgG in serum and sIgA in mucosal secretions

To determine the presence of anti-*B. mandrillaris* IgG in normal human serum and sIgA in saliva, ELISAs were

Fig. 3. Serum exhibits amoebicidal effects. *B. mandrillaris* were incubated in the presence of 20 and 100% normal human serum (NHS) or heat-inactivated serum (HI-NHS) for different lengths of time: black bars, 30 min; white bars, 1 h; grey bars, 24 h. Cell numbers and their viability were determined by haemocytometer counting and the Trypan blue exclusion assay. The counts from *B. mandrillaris* incubated alone in the absence of serum were taken as 100%, and results are presented as a percentage relative to that value. Note that 100% serum exhibited an initial amoebicidal effect, followed by an amoebistatic effect (* $P < 0.05$ using Student's *t* test with paired, two-tailed distribution). Results are representative of three independent experiments performed in triplicate; bars represent standard error.

performed. As shown in Fig. 5, serum and saliva exhibited the presence of anti-*B. mandrillaris* IgG and sIgA, respectively. Serum and saliva samples reacted specifically with *B. mandrillaris* in a dose-dependent manner (Fig. 5). Samples from three different individuals were tested and exhibited similar results (representative data are shown in Fig. 5).

Western blots reveal that serum antibodies react with several *B. mandrillaris* antigens

Earlier studies have shown that normal human serum possesses anti-*B. mandrillaris* antibodies (Huang *et al.*, 1999). To determine whether human serum possesses antibodies that react with *B. mandrillaris* antigens, Western blotting assays were performed. The findings revealed that normal human serum reacted with several *B. mandrillaris* antigens with approximate molecular masses of 148, 115, 82, 67, 60, 56, 44, 42, 40 and 37 kDa (Fig. 6).

With the growing HIV pandemic, it is reasonable to predict increasing numbers of opportunistic infections. This is particularly worrying in developing countries where HIV patients have no or limited access to novel antiretroviral therapies. Thus there is a need for continued efforts to (i) increase awareness, (ii) develop rapid diagnostic methods and (iii) understand the basic molecular mechanisms of host-parasite interactions, which should help design preventative and/or therapeutic strategies. Among

Fig. 4. Serum exhibits amoebistatic effects. *B. mandrillaris* were incubated with HBMEC (as food source) in the presence of 20 or 100% human serum for different lengths of time: black bars, 1 h; white bars, 24 h. As indicated in Fig. 3, 100% serum exhibited an initial amoebicidal effect. However, the remaining subpopulation of *B. mandrillaris* remained static over longer incubations, even in the presence of a food source, i.e. HBMEC. The counts from *B. mandrillaris* incubated alone in the absence of serum and HBMEC were taken as 100%, and results are presented as a percentage relative to that value. Note that 100% serum exhibited an initial amoebicidal effect, followed by an amoebistatic effect. Results are representative of three independent experiments performed in triplicate; bars represent standard error.

opportunistic protozoan pathogens, *B. mandrillaris* has gained particular attention in recent years. This is due to its ability to produce BGE in both immunocompromised and immunocompetent individuals. One of the major steps in BGE is amoebae invasion of the bloodstream followed by

Fig. 5. Serum contains *B. mandrillaris*-specific IgG. To determine whether serum contained *B. mandrillaris*-specific IgG, ELISAs were performed as described in Methods. Serum (■) and saliva (▲) samples contained *B. mandrillaris*-specific IgG and sIgA, respectively, in a dose-dependent manner, and the levels of *B. mandrillaris*-specific antibodies were similar. Results are means of three independent experiments performed in duplicate; bars represent standard error.

Fig. 6. Serum reacts with several antigens of *B. mandrillaris*. To determine whether normal human serum possesses antibodies which react with *B. mandrillaris* antigens, Western blots (upper panel) were performed as described in Methods. Our findings revealed that normal human serum reacted with *B. mandrillaris* antigens with approximate molecular masses of 148, 115, 82, 67, 60, 56, 44, 42, 40 and 37 kDa (Fig. 6). Lower panel, SDS-PAGE.

their haematogenous spread. *B. mandrillaris* entry into the CNS most likely occurs at the blood–brain barrier. Here, we studied the effects of serum on *B. mandrillaris* interactions with HBMEC, which form the blood–brain barrier. To the best of our knowledge, our findings reveal for the first time that normal human serum inhibits *B. mandrillaris* binding and subsequent cytotoxicity to HBMEC. Overall, these studies suggest that normal human serum protects HBMEC against *B. mandrillaris*, and that this property may help prevent blood–brain barrier perturbations.

Next, we determined whether the protective effects of serum against *B. mandrillaris* are targeted via distinct molecular mechanisms or are simply an effect secondary to the amoebistatic/amoebicidal properties of serum. We observed that normal human serum exhibited an initial limited amoebicidal effect: ~40 % of trophozoites were killed. However, a subpopulation of amoebae remained viable, although cultures were stationary over longer incubations.

The fact that serum exhibited ~50 % inhibition of amoebae binding to HBMEC (similar to amoebicidal effects) suggests that the effects of serum on the properties of *B. mandrillaris* are at least partly secondary to the amoebicidal/amoebistatic effects. This is consistent with earlier findings, which show that virulent strains of *Acanthamoeba* (a close relative of *B. mandrillaris*) resist serum-mediated killing (Toney & Marciano-Cabral, 1998).

Although BGE has been reported in immunocompetent populations (individuals who are negative for syphilis, diabetes mellitus and malignancies, and fungal, HIV-1, HIV-2 and mycobacterial infections, as well as possessing normal CD4- and CD8-positive T-lymphocyte and B-lymphocytes counts), given the rarity of the disease, it is hard to imagine that there are no predisposing factors in contracting BGE. But whether the predisposing factors are other primary infections, underlying genetic factors, exposure to an environment with widely distributed *B. mandrillaris* or a combination of the above is incompletely understood. For example, most BGE cases have occurred in the Americas (more than 90 % of cases), with the majority reported from warmer regions (Schuster & Visvesvara, 2004). Among them, a significant number of BGE cases have occurred in individuals of Hispanic origin (Schuster *et al.*, 2004). Future studies will aim to differentiate between the possibility that individuals of Hispanic origin are more exposed to *B. mandrillaris* and the possibility that they have a genetic predisposition to succumb to this disease, as suggested by Schuster *et al.* (2004).

One of the interesting findings in this study was that serum possesses antibodies that react with several *B. mandrillaris* antigens in Western blots. The antigens of *B. mandrillaris* reacted strongly with normal human serum. *B. mandrillaris* isolates from baboon tissue (ATCC 50209) and from human brain (Jayasekera *et al.*, 2005) shared several common antigens, which confirms that the two isolates are antigenically close and belong to the same species. However, the protective role of antibodies against BGE is somewhat unclear. For example, several BGE patients have been reported to possess high titres of anti-*B. mandrillaris* antibodies without a protective response, resulting in death (Huang *et al.*, 1999; Jayasekera *et al.*, 2004). This may be due to a delayed humoral response, overwhelming BGE infection, or the ability of amoebae to evade the humoral immune response. Overall, these findings confirm that normal human serum is partially inhibitory to *B. mandrillaris* properties associated with pathogenesis, but whether a healthy immune response is sufficient to control and/or eradicate these life-threatening pathogens is unknown. To this end, studies are being conducted to determine the detrimental effects of serum on *B. mandrillaris* in the presence of neutrophils/macrophages. These studies should clarify the mechanisms associated with *B. mandrillaris* pathogenesis, and this may help design preventative measures and/or develop therapeutic interventions.

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