Pathogenic *Naegleria fowleri* and non-pathogenic *Naegleria lovaniensis* exhibit differential adhesion to, and invasion of, extracellular matrix proteins

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Naegleria fowleri and *Naegleria lovaniensis* are closely related free-living amoebae found in the environment. *N. fowleri* causes primary amoebic meningoencephalitis (PAM), a rapidly fatal disease of the central nervous system, while *N. lovaniensis* is non-pathogenic. *N. fowleri* infection occurs when the amoebae access the nasal passages, attach to the nasal mucosa and its epithelial lining, and migrate to the brain. This process involves interaction with components of the host extracellular matrix (ECM). Since the ability to invade tissues can be a characteristic that distinguishes pathogenic from non-pathogenic amoebae, the objective of this study was to assess adhesion to, and invasion of, the ECM by these two related but distinct *Naegleria* species. *N. fowleri* exhibited a higher level of adhesion to the ECM components laminin-1, fibronectin and collagen I. Scanning electron microscopy revealed that *N. fowleri* attached on ECM substrata exhibited a spread-out appearance that included the presence of focal adhesion-like structures. Western immunoblotting revealed two integrin-like proteins for both species, but one of these, with a molecular mass of approximately 70 kDa, was detected at a higher level in *N. fowleri*. Confocal microscopy indicated that the integrin-like proteins co-localized to the focal adhesion-like structures. Furthermore, anti-integrin antibody decreased adhesion of *N. fowleri* to ECM components. Finally, *N. fowleri* disrupted 3D ECM scaffolds, while *N. lovaniensis* had a minimal effect. Collectively, these results indicate a distinction in adhesion to, and invasion of, ECM proteins between *N. fowleri* and *N. lovaniensis.*

INTRODUCTION

The genus *Naegleria* consists of species of free-living amoebae that are found worldwide in soil, and freshwater lakes and ponds (Marciano-Cabral & Cabral, 2007; Martinez & Visvesvara, 1997). Although numerous species from this genus have been identified in the environment and in domestic water supplies (Anderson & Jamieson, 1972; Craun *et al.*, 2005; Gyori, 2003; Jamerson *et al.*, 2009; Marciano-Cabral *et al.*, 2003; Yoder *et al.*, 2004), only one species, *Naegleria fowleri*, has been linked to disease in humans (Carter, 1968; Cerva & Novăk, 1968; Martinez, 1985). *N. fowleri* is the causative agent of primary amoebic meningoencephalitis (PAM), a rapidly fatal disease of the central nervous system (CNS) that occurs in individuals who have been engaged recently in activities such as swimming or diving in warm freshwater bodies (Carter,

1968; Martinez, 1985). Infection occurs when amoebae enter the nasal passages and attach to the nasal mucosa (Carter, 1972; Martinez, 1985). Following contact with the nasal epithelium, amoebae penetrate the underlying basement membrane and migrate to the brain, wherein they multiply and destroy tissues, resulting in haemorrhagic necrosis and death (Jarolim *et al.*, 2000; Martinez *et al.*, 1973; Martinez, 1985). While the attachment process may induce an innate immune response, *N. fowleri* appears to be resistant. For example, it has been reported that it activates the complement system but is capable of evading complement-mediated lysis (Marciano-Cabral & Cabral, 2007).

A pivotal step during infection by *N. fowleri* is its interaction with the host basement membrane, a complex layer of specialized extracellular matrix (ECM) glycoproteins and proteoglycans that serves to separate the epithelium from stromal tissues (Jarolim *et al.*, 2000; LeBleu *et al.*, 2007). During migration to the brain, *N. fowleri* must transverse the epithelium and contact specific ECM components, such as laminin-1, an important component of the basement

membrane (Yurchenco & Schittny, 1990), collagen I, a prevalent ECM component in connective tissues (Nimni, 1983), and fibronectin, an adhesive glycoprotein found in connective tissues and the blood (Hynes & Yamada, 1982).

It has been reported previously that protozoa that are pathogenic recognize components of the ECM (Gordon et al., 1993; Han et al., 2004; Rocha-Azevedo et al., 2007, 2009; Shibayama et al., 2003). For example, Entamoeba histolytica binds to ECM components, an interaction that may play an important role in its penetration of the intestinal mucosa (de Lourdes Muñoz et al., 2001; Li et al., 1995; Talamás-Rohana & Meza, 1988). Species of Acanthamoeba have been reported to bind to laminin-1, collagen IV and fibronectin (Gordon et al., 1993). However, pathogenic Acanthamoeba culbertsoni has been shown to exhibit a higher level of attachment to ECM components than non-pathogenic Acanthamoeba astronyxis (Rocha-Azevedo et al., 2009). A 39 kDa surface-associated fibronectin-binding protein has been identified for Trichomonas vaginalis and linked to colonization and persistence of infection (Alderete et al., 2002; Lama et al., 2009). Also, a 67 kDa laminin-binding protein has been identified in Leishmania donovani, the causative agent of visceral leishmaniasis (Bandyopadhyay et al., 2001). Consistent with these observations, it has been reported that N. fowleri binds to immobilized fibronectin in a concentration-dependent manner through the mediation of a 60 kDa fibronectin-binding protein (Han et al., 2004).

In the present study the binding and invasive properties of thermotolerant pathogenic N. fowleri were compared with those of a thermotolerant non-pathogenic species, Naegleria lovaniensis. Adhesion to ECM components was shown to differ between the two species, with a higher level of adhesion observed for N. fowleri. Scanning electron microscopy (SEM) revealed differences in morphology of N. fowleri when compared with N. lovaniensis. N. fowleri exhibited a spread-out appearance that was associated with the presence of focal adhesion-like extensions. Western immunoblots revealed the presence of two protein species that were immunoreactive with an anti-integrin antibody. The larger of these, a 70 kDa integrin-like protein, was found at higher levels for N. fowleri as compared with N. lovaniensis. Confocal microscopy revealed that integrin-like proteins co-localized with the focal adhesion-like structures on N. fowleri. In addition, an anti-integrin antibody decreased adhesion of N. fowleri to ECM components. Finally, using an in vitro model of the ECM, N. fowleri exhibited enhanced transmigration as compared with N. lovaniensis. These results, while limited in scope to assessment of two species within the same genus, suggest that the ability to adhere to, and invade, ECM scaffolds serves as a phenotypic marker for differentiating pathogenic from non-pathogenic Naegleria.

METHODS

Naegleria cultures. N. fowleri (ATCC 30894) and N. lovaniensis (ATCC 30569) were obtained from the American Type Culture Collection and were grown at 37 °C for 24 h in Oxoid medium in 75 cm^2 plastic flasks (Cline et al., 1983). A mouse-passaged strain of N. fowleri was utilized in these studies to maintain their virulence (Toney & Marciano-Cabral, 1992). For experiments, amoebae were detached from flasks by bumping and washed two times in 0.01 M PBS, pH 7.2.

ECM surface coating. Twenty-four-well plates or glass coverslips were coated with Sigmacote (Sigma), washed once with deionized water and air-dried overnight at room temperature. The surfaces of plates or coverslips were then incubated (2 h, 37 °C) with fibronectin from human plasma (Sigma), collagen I from rat tails (Sigma) or laminin-1 from Engelbreth-Holm Swarm mouse sarcoma (Invitrogen) at 50 µg ml^{-1} diluted in PBS. Before addition of amoebae, wells or coverslips were washed gently once with PBS to remove unattached ECM glycoproteins.

Attachment assay. N. fowleri and N. lovaniensis were radiolabelled (24 or 48 h) with 60 µCi (2.22 MBq) [^{3}H]uridine [specific activity: 35.6 Ci mmol^{-1} (1317.2 GBq mmol^{-1})] (Toney & Marciano-Cabral, 1992). These labelled amoebae then were detached from flasks, washed two times with PBS, and counted using a haemocytometer. Amoebae (2×10^5) then were added to ECM-coated wells (50 µg µl^{-1}) for 50 min at 37 °C. Following incubation, wells were washed once with PBS to remove non-adherent amoebae. Wells coated with BSA were used as a control for non-specific attachment. Attached amoebae were solubilized using 2 % (v/v) Triton X-100 to release the incorporated radiolabel, which was quantified by liquid scintillation counting (Packard 2200CA Tri-Carb Liquid Scintillation Analyzer, Packard Instrument). c.p.m. were converted to percentage attachment, where 100 % represented the amount of radioactivity present in 2×10^5 amoebae. Percentage attachment was defined using the following formula: percentage attachment=(c.p.m. of attached amoebae/c.p.m. of 2×10^5 amoebae) × 100 %.

Amoeba whole-cell lysates. Amoebae grown for 24 h were detached from tissue culture flasks, washed twice in PBS, and disrupted by three cycles of freezing in liquid nitrogen and thawing at 37 °C in lysis buffer containing protease inhibitors (50 mM Tris/HCl, pH 7.4, 1 mM PMSF, 1.5 mM pepstatin A and 1.5 mM leupeptin). Cell lysates were used for Western immunoblotting analysis.

Amoeba plasma membrane isolation. Amoebae grown for 24 h were detached from tissue culture flasks and washed twice in PBS, and membrane proteins were isolated using the Mem-PER eukaryotic membrane protein extraction kit (Pierce), according to the manufacturer's instructions.

Western immunoblot analysis. Protein concentrations in amoeba whole-cell lysates (30 µg) and membrane fractions (5 µg) were quantified by the Bradford method (Bradford, 1976) and the RC/DC protein assay (Bio-Rad), respectively. These then were subjected to 12 % polyacrylamide SDS-PAGE. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Towbin et al., 1979). The membranes were rinsed (5 min) in Tris-buffered saline containing 0.1 % tween 20 (TBST) and treated for 1 h at room temperature with blocking buffer consisting of 5 % (w/v) non-fat dry milk in TBST. Nitrocellulose membranes were then rinsed and incubated overnight with chicken polyclonal antibody directed against human $\beta1$ integrin subunit (GW22754, Sigma) diluted in blocking buffer (whole-cell lysates 1 : 500, membrane fractions 1 : 1000). Membranes were washed six times (5 min each) in TBST and incubated with a peroxidase-conjugated rabbit anti-chicken antibody (diluted 1 : 10 000). Protein

bands were visualized using a chemiluminescence detection kit (Perkin Elmer), according to the manufacturer's instructions. A U937 human monocyte lymphoma (ATCC CRL-1593.2) whole-cell homogenate (10 µg) or membrane fraction (5 µg) served as a positive control for integrin protein. The chicken polyclonal antibody directed against the human $\beta 1$ integrin subunit used in Western immunoblots was pre-adsorbed with U937 cells (5×10^6) prior to use as a negative control. Image analysis of immunoreactive bands was performed using Quantity One version 4.5 software (Bio-Rad Laboratories).

Confocal microscopy. *N. fowleri* and *N. lovaniensis* amoebae were incubated on uncoated, collagen-I-coated, laminin-1-coated or fibronectin-coated coverslips (25 min, 37 °C), fixed in 4 % para-formaldehyde (60 min, 37 °C) and rinsed with Dulbecco's PBS (DPBS; containing Ca^{2+} and Mg^{2+}). Cells were permeablized (20 min) with 0.1 % Triton X-100 in DPBS, blocked (60 min) with 5 % BSA in DPBS, and incubated (2 h, room temperature) with a FITC-conjugated monoclonal anti-$\beta 1$ integrin subunit antibody (1 : 250, P4G11, Millipore). Coverslips were washed with DPBS (1 % BSA), incubated with Alexafluor 594 phalloidin (1 : 500, A12381, Invitrogen) and washed with DPBS. Coverslips were incubated with DAPI (1 : 20 000) to identify nuclei. Additionally, surface staining was assessed with the omission of the permeabilization step. Amoebae were fixed in 4 % paraformaldehyde (60 min, 37 °C) and rinsed with DPBS. Cells were blocked (60 min) with 5 % BSA and 10 % normal goat serum in DPBS, and incubated (2 h, room temperature) with a FITC-conjugated monoclonal anti-$\beta 1$ integrin subunit antibody (1 : 250, P4G11, Millipore). Amoebae also were probed with a FITC-conjugated monoclonal anti-human IgG antibody (1 : 250, 054211, Invitrogen) as a negative control. Images were acquired by spinning-disk confocal microscopy using a BX51 microscope (Olympus) affixed with an Olympus disk spinning unit and an Orca-R2 CCD camera (Hamamatsu). Images were processed using the Slidebook software package (Intelligent Imaging Innovations).

Competitive attachment assays. *N. fowleri* amoebae were radiolabelled (24 h) with 60 µCi (2.22 MBq) [³H]uridine [specific activity: 35.6 Ci mmol^{-1} (1317.2 GBq mmol^{-1})] (Thong & Ferrante, 1986). Following labelling, amoebae were pre-incubated (20 min) with the peptide sequence Gly-Arg-Gly-Asp-Thr-Pro (500 µg ml^{-1}, MP Biomedicals), which contains an integrin recognition site, to determine whether the peptide could inhibit attachment (Ruoslahti, 1996). Amoebae were also incubated (20 min) with the peptide sequence Ser-Asp-Gly-Arg-Gly (Sigma), which represents a non-specific control. Amoebae were then used in the attachment assay using collagen I. c.p.m. were converted to percentage attachment, where 100 % represented the amount of radioactivity present in 2×10^5 amoebae. Percentage attachment was defined using the formula: percentage attachment=(c.p.m. of attached amoebae)/ (c.p.m. of 2×10^5 amoebae) $\times$ 100 %. Additionally, a second competitive attachment assay was performed using a blocking antibody. *N. fowleri* (2×10^5) was preincubated (10 min) with a monoclonal anti-$\beta 1$ integrin subunit antibody (P4C10) (1 : 100) or with the irrelevant antibody anti-keyhole limpet haemocyanin (KLH; KLH12B4.G3.A8, Abcam) (1 : 100) used as a control, prior to addition to ECM components for 50 min to assess for blocking of attachment. At the end of the incubation period, unattached amoebae were collected, fixed with glutaraldehyde and counted using a haemocytometer to determine whether the anti-$\beta 1$ integrin antibody inhibited attachment.

Invasion assays. Tissue culture inserts (Greiner BioOne) with a pore-size of 8 µm were coated (100 µl, 10 min) with either type I collagen (1.7 mg ml^{-1}) or Matrigel (BD Biosciences) (1.7 mg ml^{-1}), a reconstituted basement membrane solution which forms a matrix that has been used extensively for *in vitro* invasion studies (Kleinman

& Jacob, 2001; Kleinman & Martin, 2005). Following the coating period, residual ECM solutions were removed, and the inserts were allowed to dry (2 h) at room temperature. The coated inserts were placed in 24-well plates and were used as an upper chamber. Oxoid medium was added to the bottom chamber of the tissue culture well to serve as an amoeba attractant. *N. fowleri* or *N. lovaniensis* (2×10^5 amoebae) suspended in PBS were added to the upper chamber, and the plates were incubated for 4 h at 37 °C. Amoebae that passed through the ECM-coated inserts and into the bottom chamber were collected and counted using a haemocytometer.

SEM. To examine for attachment to ECM components, *N. fowleri* and *N. lovaniensis* were incubated (50 min) on coated glass coverslips as described in the attachment assay and then fixed with 2.5 % glutaraldehyde. To examine for invasion of the ECM matrix, *N. fowleri* and *N. lovaniensis* were incubated (2 h) at 37 °C on collagen I or Matrigel (5 mg ml^{-1})-coated tissue culture inserts. Inserts then were immersed (1 h) in 2.5 % (v/v) glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, washed four times with PBS, and treated (40 min in the dark) with 2 % (w/v) osmium tetroxide buffered in 0.1 M cacodylate buffer, pH 7.2. The inserts were washed with PBS, dehydrated in a graded series of ethanol solutions, subjected to critical-point drying with CO_2 as the transitional fluid, mounted on stubs and coated with gold (30 nm) (Rocha-Azevedo *et al.*, 2007). Samples were examined with a Zeiss EVO 50XVP scanning electron microscope operating at an accelerating speed of 15 kV.

Statistical analysis. Data were expressed as the mean$\pm$SD of the mean. To determine statistical significance ($P<0.05$), a two-tailed, unpaired Student's *t* test and one-way analysis of variance (ANOVA) tests were used.

RESULTS

Naegleria attachment on ECM glycoproteins

The attachment of *Naegleria* on collagen I, fibronectin and laminin-1 was assessed. *N. fowleri* and *N. lovaniensis* demonstrated a differential pattern of adhesion (Fig. 1). While a low level of attachment on BSA-coated wells was observed, *N. fowleri* exhibited a significantly higher level ($P<0.05$, ANOVA and Student's *t* test) of attachment when exposed to laminin-1, collagen I or fibronectin. *N. lovaniensis* exhibited minimal differences in attachment to collagen I and laminin-1 compared with the BSA control. However, a slight increase ($P<0.05$, ANOVA and Student's *t* test) in attachment of *N. lovaniensis* to fibronectin was observed. While both species exhibited attachment to fibronectin, attachment for *N. fowleri* was 30 % higher than that to the BSA control, while that of the non-pathogen *N. lovaniensis* was 10 % higher than that to the BSA control (Fig. 1a, b). Similarly, attachment to collagen I and laminin-1 was increased by approximately 25 and 20 %, respectively, for *N. fowleri* as compared with BSA (Fig. 1a). However, attachment of *N. lovaniensis* to laminin-1 and collagen I did not differ from the level for the BSA control (Fig. 1b). These results indicate a significantly higher level of adhesion to ECM components by *N. fowleri* as compared with *N. lovaniensis*.
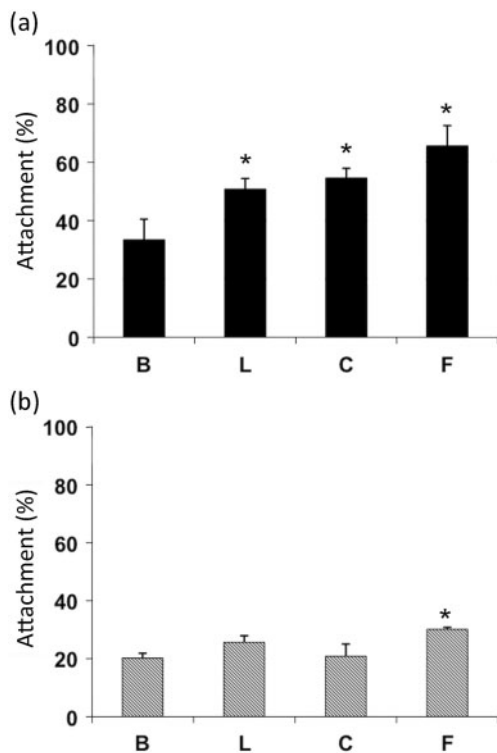
Fig. 1. Attachment of (a) *N. fowleri* and (b) *N. lovaniensis* on ECM glycoproteins. Radiolabelled amoebae were added to tissue culture wells coated with 50 µg ml^{-1} laminin-1 (L), collagen I (C) or fibronectin (F). Tissue culture wells treated with BSA (B) served as a control. The number of amoebae added to each well was 2×10^5, which represents 100 %. Experiments were performed twice in triplicate. Asterisks represent $P<0.05$ by ANOVA with Student's *t* test as compared with BSA.

Morphological assessment of *Naegleria* on ECM substrata

SEM demonstrated morphological differences between the two species upon exposure to ECM components. *N. fowleri* demonstrated a spread-out appearance on collagen I, laminin-1 and fibronectin (Fig. 2b–d). Furthermore, these amoebae when exposed to ECM components exhibited lamellipodia and morphological features that were similar to those of focal adhesions (Fig. 2e, f, arrows). These features were not observed when *N. fowleri* was subjected to adherence onto non-coated glass surfaces (Fig. 2a). In contrast, *N. lovaniensis* exhibited an elongated form when placed on all substrata (Fig. 3a–d). Focal adhesion-like structures were not observed for *N. lovaniensis* when placed on any of the substrata (Fig. 3).

Detection of integrin-like proteins

Western immunoblot analysis was performed on whole-cell lysates of *N. fowleri* and *N. lovaniensis* using a polyclonal antibody directed to a human $\beta1$ integrin subunit. A band

of approximately 53 kDa was detected for both *N. fowleri* and *N. lovaniensis* (Fig. 4a). Image analysis of the band yielded optical density units of 166 and 144 for *N. fowleri* and *N. lovaniensis,* respectively. However, an additional band of approximately 70 kDa was observed at a greater than fourfold higher level for *N. fowleri* (Fig. 4). For *N. fowleri*, approximately 149 optical density units were obtained as compared with 34 optical density units for *N. lovaniensis.* Additionally, Western immunoblot analysis performed on membrane fractions of *N. fowleri* and *N. lovaniensis* yielded protein bands of approximately 53 and 70 kDa (Fig. 4b). The protein band of approximately 70 kDa was observed at a greater than 1.55-fold higher level for *N. fowleri* (Fig. 4b). Pre-adsorption of the anti-$\beta1$ integrin subunit antibody was performed prior to Western immunoblot analysis on membrane fractions using whole U937 cells. Pre-adsorption resulted in a 1.5-fold decrease in the 70 kDa protein band and total abolition of the 53 kDa protein band (Fig. 4b). Confocal microscopy confirmed the presence of integrin-like proteins on *N. fowleri* and *N. lovaniensis.* Images shown (Fig. 5) are for interaction with collagen I, but are representative of *Naegleria* placed on all three ECM components. Both species exhibited $\beta1$ integrin reactivity within the cell body that was dispersed in a punctate pattern (Fig. 5b, e). However, a distinctive pattern of distribution was observed for *N. fowleri*. These amoebae exhibited a co-localization of actin filaments and $\beta1$ integrin-like protein at their leading edge (arrow) and at focal adhesion-like structures (Fig. 5a, c). This pattern of co-localization was not observed for *N. lovaniensis* (Fig. 5d, f). Additionally, confocal microscopy was performed in the absence of the permeabilization step (0.1 % Triton X-100) to determine whether the integrin-like molecules were present on the surface of the amoebae. Both species exhibited surface $\beta1$ integrin reactivity (Fig. 6a, c), consistent with integrin-like molecules being located on the cell surface. However, this surface reactivity indicative of the presence of $\beta1$ integrin-like proteins was found to be concentrated at the focal adhesion-like structures of *N. fowleri*. A lack of reactivity was observed when the amoebae were probed with a mouse anti-human IgG antibody, which served as a negative control (Fig. 6b, d). Since Western immunoblot analysis and confocal microscopy suggested the presence of an integrin-like protein on amoebae, an RGD-containing peptide (Gly-Arg-Gly-Asp-Thr-Pro) with an integrin binding site was used to assess its ability to inhibit adhesion to collagen I. This peptide, when used in a competitive binding assay, however, did not inhibit adhesion of *N. fowleri* to collagen I (results not shown). To further investigate whether an integrin-like protein was linked functionally to *N. fowleri* attachment, a blocking antibody was utilized in *N. fowleri* attachment inhibition studies (Table 1). Similar studies were not performed using *N. lovaniensis*, since this species exhibited minimal attachment to laminin-1, collagen I and fibronectin. Adhesion of *N. fowleri* on laminin-1, collagen I and fibronectin was inhibited by the $\beta1$ integrin antibody. In the absence of $\beta1$ integrin antibody, 75 % of input *N.*
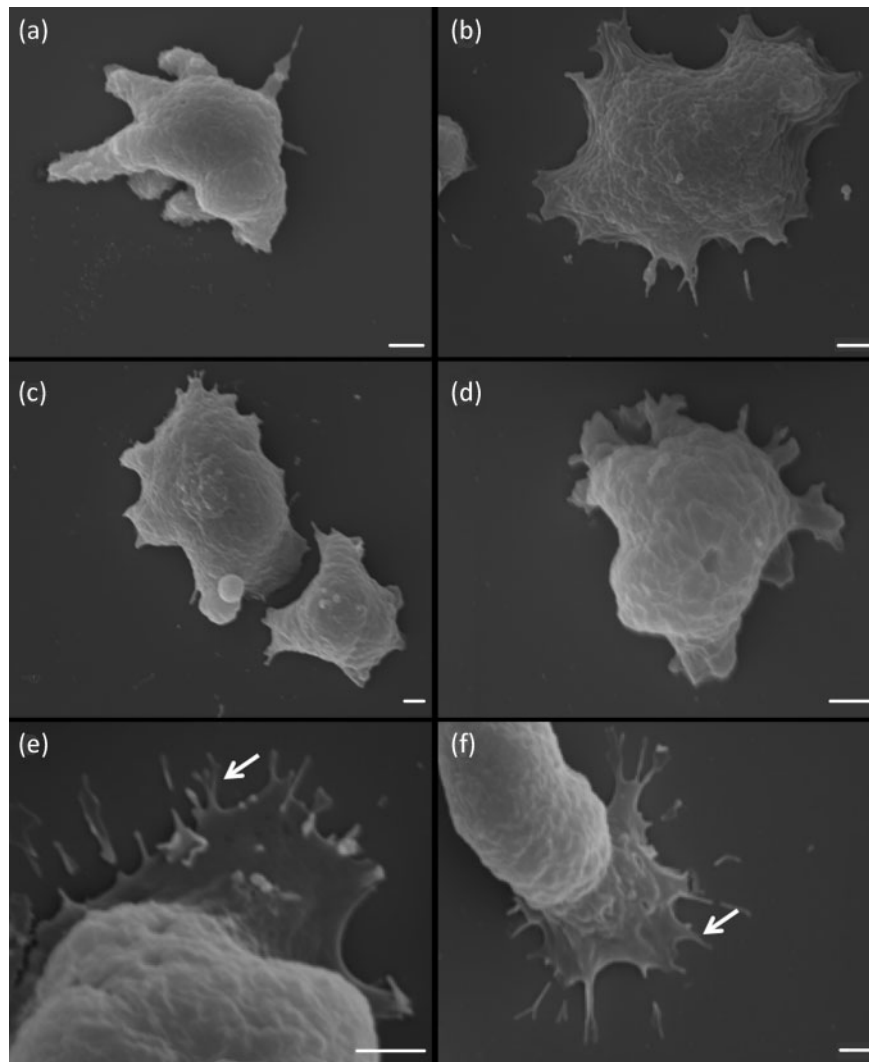
Fig. 2. Scanning electron micrographs of *N. fowleri* attached to various substrata. Amoebae interacting with (a) glass or on ECM glycoproteins, (b) collagen I, (c) fibronectin and (d) laminin-1. (e, f) Higher magnification of the focal adhesion-like structures on laminin-1 (arrows). Bars, 2 μm.

fowleri were found to bind to laminin-1 and collagen I. Also, 80 % of input *N. fowleri* were found to bind to fibronectin. In contrast, in the presence of anti-β1 antibody, 40 and 30 % of input *N. fowleri*, respectively, were found to bind to laminin-1 and collagen I. Of input *N. fowleri*, 50 % were found to bind to fibronectin in the presence of anti-β1 integrin. Thus, in the presence of the anti-β1 integrin antibody, 1.4- to 2.2-fold less binding was obtained as compared with that obtained in the presence of the integrin-irrelevant anti-KLH antibody.

Invasion on 3D collagen I or Matrigel

SEM indicated that *N. fowleri* amoebae passed through collagen I and Matrigel (Fig. 7). Evidence for penetration of these matrices was obtained as early as after a 2 h incubation period. In contrast, a very low number of *N. lovaniensis* was observed to have disrupted the ECM and, then, only after 4 h of incubation. SEM revealed a remodelling of collagen I fibres, with overt extensive damage to the matrix following 2 h of exposure to *N. fowleri* (Fig. 8a). Large gaps in the matrix were observed that appeared to provide a passage for *N. fowleri* through the collagen I layer. No observable perturbation of the matrix was observed in the presence of *N. lovaniensis* (Fig. 8b). However, both *Naegleria* species exhibited the presence of 'food-cups' when in the presence of the collagen I matrix (Fig. 8). Similar results were obtained for *N. fowleri* versus *N. lovaniensis* when placed on Matrigel. *N. fowleri* exerted extensive damage to the Matrigel matrix as evidenced by the presence of large holes or gaps (Fig. 8c). In contrast, the Matrigel layer remained relatively intact when exposed (2 h) to *N. lovaniensis* (Fig. 8d).
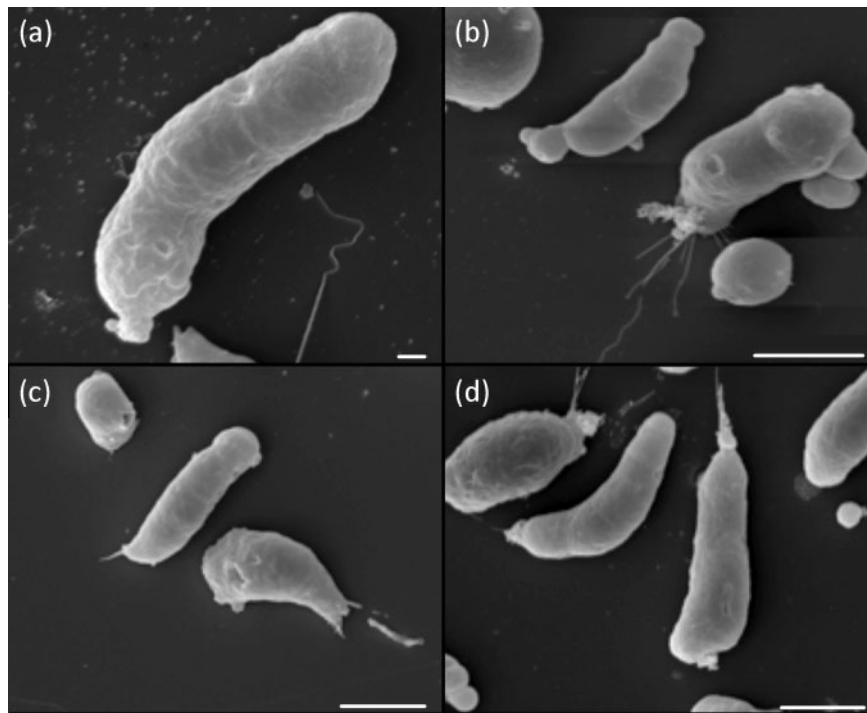
Fig. 3. Scanning electron micrographs of *N. lovaniensis* placed on various substrata. Amoebae interacting on (a) glass or on ECM glycoproteins, (b) collagen I, (c) fibronectin and (d) laminin-1. Bars: (a), 1 μm; (b–d), 10 μm.
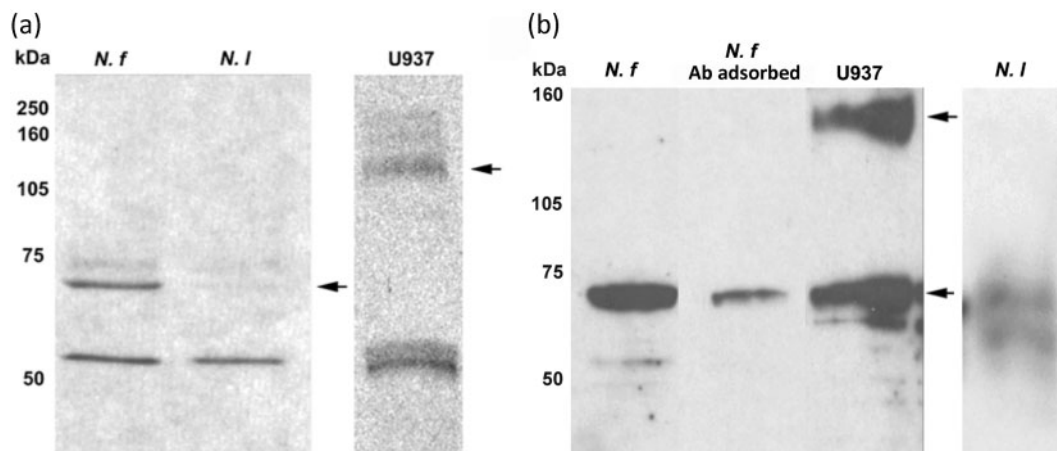

Fig. 4. Western immunoblot analysis of (a) *N. fowleri* (*N. f*) and *N. lovaniensis* (*N. l*) whole-cell lysates and (b) *N. fowleri* and *N. lovaniensis* membrane fractions for the detection of integrin-like proteins. Amoebic extracts were separated by 12 % SDS-PAGE and transferred to a nitrocellulose membrane (left panel). A human leukaemic monocyte lymphoma (U937) cell extract was used as a positive integrin control. Membranes then were incubated with a polyclonal chicken anti-human β1 integrin subunit antibody followed by horseradish peroxidase-conjugated rabbit anti-chicken antibody. The chicken polyclonal antibody directed against the human β1 integrin subunit used in Western immunoblotting was pre-adsorbed with U937 cells prior to use to serve as a negative control (*N. f* Ab adsorbed). Arrows indicate the 70 kDa (*N. fowleri* and *N. lovaniensis*) and 140 kDa (U937) major immunoreactive bands.
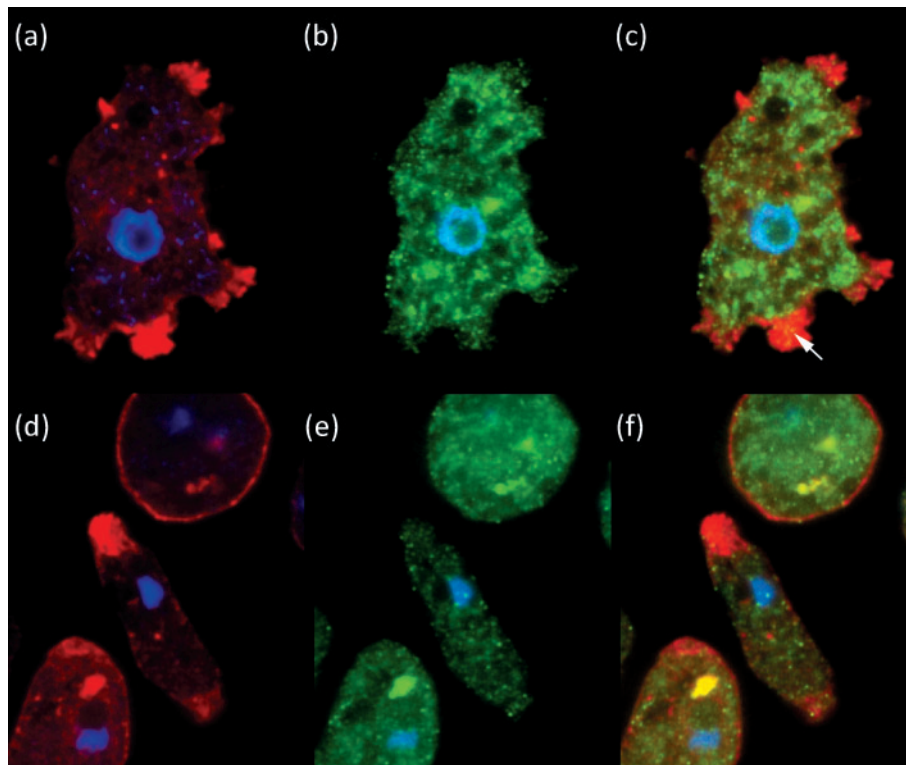
Fig. 5. Confocal micrographs of *N. fowleri* and *N. lovaniensis* placed on collagen I. *N. fowleri* was probed with Alexafluor 594 phalloidin (a) or FITC-conjugated monoclonal anti-β1 integrin antibody (b). The merged fluorescent image is shown in (c). *N. lovaniensis* was probed with Alexafluor 594 phalloidin (d) or FITC-conjugated monoclonal anti-β1 integrin antibody (e). The merged fluorescent image is shown in (f). Nuclear localization is depicted in all panels by DAPI staining (a–f). All images are magnified ×100.

DISCUSSION

N. fowleri is the only species of the genus *Naegleria* that has been isolated from fatal CNS infections in humans (Martinez, 1985; Yoder *et al.*, 2010). Other species, including *Naegleria italica* and *Naegleria australiensis*, have been shown to cause disease in experimentally infected mice but have never been linked to human infections (De Jonckheere, 2004). The ability to elicit cytopathic effects on cells in culture does not appear to define pathogenicity for *Naegleria*. That is, while species of *Naegleria* such as *N. lovaniensis* and *Naegleria gruberi* destroy mammalian cells *in vitro* (Marciano-Cabral *et al.*, 1982; Marciano-Cabral & Fulford, 1986), they have yet to be shown to be causative of human disease. Also, the ability to tolerate temperatures of 37 °C or higher does not appear to be a defining marker of pathogenicity for *Naegleria*. Thermotolerance is not seen with *N. gruberi*, and it has been proposed that this lack of heat tolerance results in humans and other mammals, such as mice, being non-permissive hosts (Marciano-Cabral & Fulford, 1986). Furthermore, *N. lovaniensis* is thermotolerant and can survive at temperatures of 37 °C and higher (Stevens *et al.*, 1980), yet has not been associated with human infection.

The interaction of cells with ECM components plays an important role in mediating cell adhesion and migration (Berrier & Yamada, 2007), and promoting invasion of host tissue. Binding to, and passage through, ECM components by micro-organisms have been implicated in the pathogenesis of bacterial, viral, protozoan and fungal infections (Alderete *et al.*, 2002; Boshuizen *et al.*, 2004; de Bentzmann *et al.*, 2004; Gozalbo *et al.*, 1998; Hostetter, 1999; Kottom *et al.*, 2008; Lama *et al.*, 2009; Casta e Silva Filho *et al.*, 1988). In this context, the ability of *N. fowleri* to adhere to and invade ECM components may serve as a hallmark of pathogenicity. For example, attachment to the nasal epithelium by *N. fowleri* has been reported to be an important early event in the pathogenesis of PAM (Martinez *et al.*, 1973). Studies characterizing experimentally induced PAM in mice have shown that deep invasion occurs after *N. fowleri* amoebae penetrate the basement membrane (Martinez *et al.*, 1973).

In the present study, two species within the genus *Naegleria*, one linked to pathogenicity in humans (Carter, 1968; Cerva & Novăk, 1968; Martinez, 1985) and the other a non-pathogen (Stevens *et al.*, 1980; Marciano-Cabral & Fulford, 1986), were selected for assessment of differences
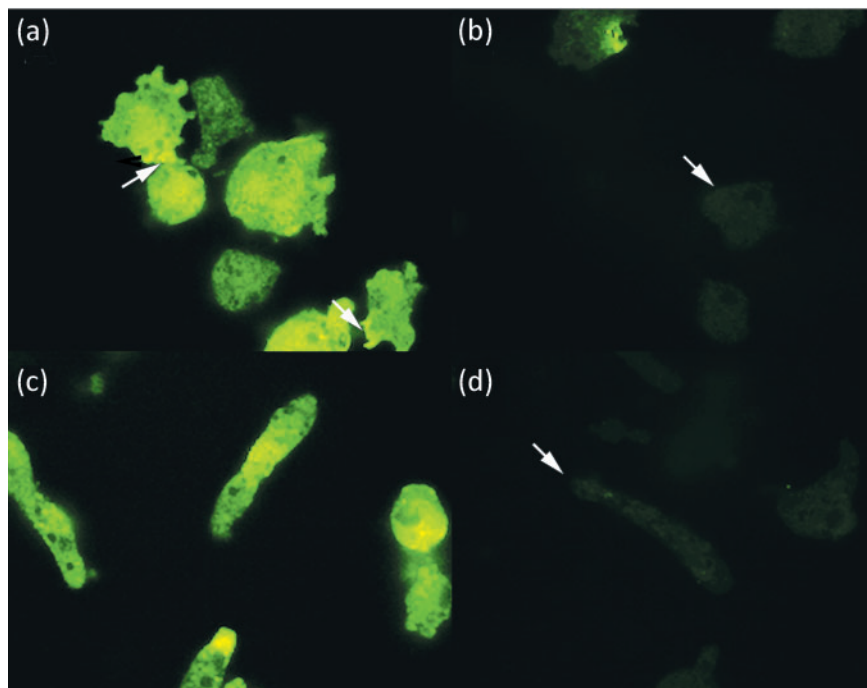
Fig. 6. Confocal micrographs of surface localization on *N. fowleri* and *N. lovaniensis* placed on collagen I. *N. fowleri* was probed with FITC-conjugated monoclonal anti-β1 integrin antibody (a) or FITC-conjugated monoclonal anti-human IgG antibody (b). *N. lovaniensis* was probed with FITC-conjugated monoclonal anti-β1 integrin antibody (c) or FITC-conjugated monoclonal anti-human IgG antibody (d). All images are magnified ×100. The arrows in (a) indicate concentrated localization of β1 integrin immune reactivity at focal adhesion-like structures of *N. fowleri*. The arrows in (b) and (d) indicate the location of amoebae in the negative control that was probed with a FITC-conjugated monoclonal anti-human IgG antibody.

in attachment to, and invasion of, ECM components. A differential level of binding to laminin-1, collagen I and fibronectin was observed for *N. fowleri* versus *N. lovaniensis*. Whereas *N. lovaniensis* exhibited a level of attachment to the different ECM components that did not differ in large measure from that noted for the BSA control, a significant level of attachment on all matrices was observed for *N. fowleri* when compared with the BSA control. These observations indicate that, in contrast to *N.*

lovaniensis, *N. fowleri* 'recognizes' select binding motifs on the target ECM proteins. Thus, in order to gain further insight into the mode by which *N. fowleri* attaches to ECM components, SEM was performed. *N. fowleri* exhibited a spread-out appearance on ECM composites and displayed numerous focal adhesion-like extensions that were in contact with, and apparently attached to, sites on collagen I, fibronectin and laminin-1. Confocal microscopy demonstrated that β1 integrin and actin immunoreactivity

Table 1. Inhibition of attachment of *N. fowleri* to ECM components

N. fowleri (2×10^5) was preincubated (10 min) with anti-KLH or monoclonal anti-β1 integrin antibody prior to addition to ECM components for 50 min. Unattached amoebae were then collected, fixed with glutaraldehyde and counted using a haemocytometer. The percentage of bound amoebae was calculated using the following formula: percentage bound=[(2×10^5 amoebae)−(number of amoebae recovered)/(2×10^5 amoebae)] × 100 %. One hundred per cent bound would indicate no amoebae recovered.

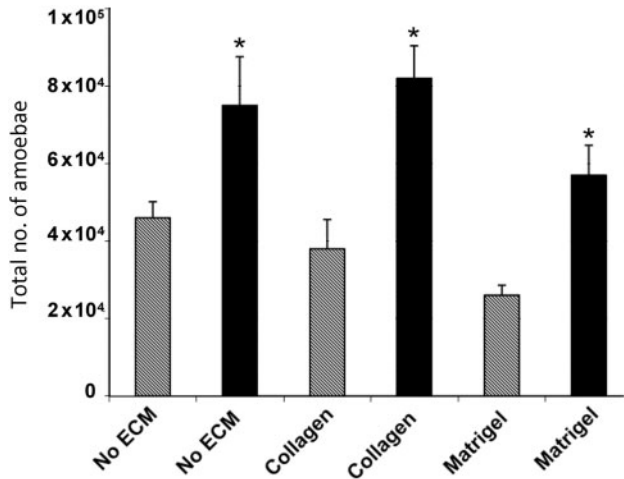| ECM component	No antibody		Anti-KLH		Anti-β1 integrin	
	Amoebae recovered	Percentage bound	Amoebae recovered	Percentage bound	Amoebae recovered	Percentage bound
Laminin-1	5.0×10^4	75 %	7.0×10^4	65 %	1.2×10^5	40 %
Collagen I	5.0×10^4	75 %	7.0×10^4	65 %	1.4×10^5	30 %
Fibronectin	4.0×10^4	80 %	6.0×10^4	70 %	1.0×10^5	50 %

Fig. 7. Invasion of *N. fowleri* and *N. lovaniensis* through ECM scaffolds (collagen I and Matrigel). Amoebae that invaded were counted using a haemocytometer. Black bars, *N. fowleri*; grey bars, *N. lovaniensis*. Experiments were performed twice in triplicate. Asterisks represent $P<0.05$ by Student's t test.

co-localized at these amoebic extensions, consistent with their designation as focal adhesion-like structures (Burridge & Chrzanowska-Wodnicka, 1996). These morphological features were not observed for *N. lovaniensis*. Additionally, confocal microscopy and SEM demonstrated that *N. lovaniensis* maintained a rounded appearance when placed on ECM composites and did not exhibit focal adhesion-like structures. It has been reported that mammalian cell attachment triggers signalling that leads to formation of focal adhesions (Burridge & Chrzanowska-Wodnicka, 1996). Such focal adhesions, or cell matrix adhesions, as large macromolecular aggregates, appear to serve both as anchorage points for cells and as 'sensors' of ECM components (Riveline *et al.*, 2001). Consistent with these observations, it has been shown that pathogenic amoebae, such as *N. fowleri* and *E. histolytica*, form actin plates upon exposure to ECM components that are involved in adhesion (Han *et al.*, 2004; Talamás-Rohana *et al.*, 1994).

Formation of focal adhesions generally involves integrins that typically are found on mammalian cells and are involved in recognition of an ECM motif (Ruoslahti, 1996). In this structural rearrangement model, integrins link the extracellular environment with the actin cytoskeleton (Burridge &
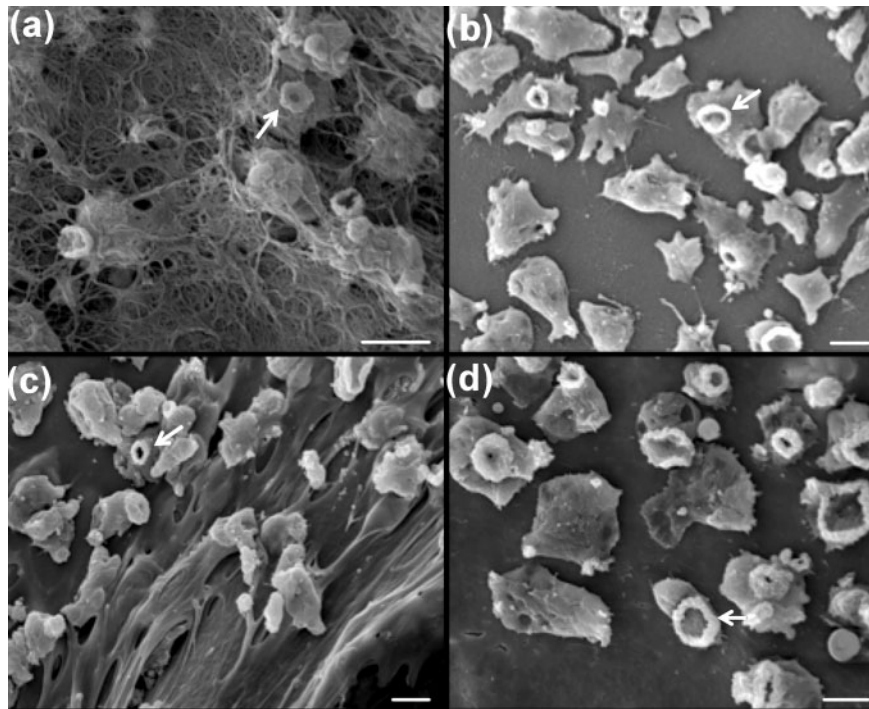

Fig. 8. Interaction (2 h) of *N. fowleri* and *N. lovaniensis* with collagen I and Matrigel matrices. (a) *N. fowleri* emerging through the bottom side of the collagen I scaffold and (b) *N. lovaniensis* interacting with collagen I on the surface of the scaffold. (c) *N. fowleri* interacting with the Matrigel surface. (d) *N. lovaniensis* interacting with the Matrigel surface. Note the presence of 'food-cups' (arrows) on both *N. fowleri* and *N. lovaniensis*. Bars, 10 μm.

Chrzanowska-Wodnicka, 1996). Many microbial pathogens express integrin-like molecules on their surface. For example, a 140 kDa β1 integrin-like molecule has been identified in *E. histolytica* and has been linked to attachment to fibronectin (Sengupta *et al.*, 2001; Talamás-Rohana & Meza, 1988; Talamás-Rohana *et al.*, 1994). Furthermore, binding of this β1 integrin-like molecule to fibronectin has been demonstrated to trigger a signal transduction cascade in *E. histolytica* involving tyrosine protein kinase, which is similar to that observed for mammalian cells (Hernández-Ramírez *et al.*, 2000; Meza, 2000; Flores-Robles *et al.*, 2003). Han *et al.* (2004) have reported that *N. fowleri* possesses an integrin-like molecule, which binds to immobilized fibronectin. This molecule was designated as an α integrin subunit and has been demonstrated to play a role in cytotoxicity. In addition, a recently published sequence of the *N. gruberi* genome has revealed the presence of a von Willebrand factor type A (VWA) domain-containing protein (Fritz-Laylin *et al.*, 2010). It has been suggested that all integrin beta subunits contain VWA domains (Tuckwell, 1999; Whittaker & Hynes, 2002).

In the present study, Western immunoblot analysis using a human β1 integrin antibody demonstrated that *N. fowleri* and *N. lovaniensis* exhibited a similar level of immune reactivity for a 53 kDa protein. However, a 70 kDa immunoreactive protein species was found at a higher level for *N. fowleri*. While the antibody used in the present study was directed at the human β1 integrin subunit, the results are consistent with reports that amoebae express integrin-like proteins. Additionally, Western immunoblot analysis using membrane fractions demonstrated that these proteins localized to the surface of the amoebae, as has been demonstrated for mammalian integrins and other eukaryotes. For example, an adhesion molecule with β integrin features has been identified in free-living *Dictyostelium* amoebae (Cornillon *et al.*, 2006). However, the mere presence of cell surface integrin-like molecules may not be a distinction that discriminates pathogenic from non-pathogenic amoebae. For example, both *E. histolytica*, a pathogen, and *Entamoeba dispar*, a non-pathogen, have been reported to express integrin-like proteins on their surface (Pillai & Kain, 2005). Similarly, *Candida* spp. have been reported to express integrin-like proteins that mediate binding to fibronectin (Santoni *et al.*, 1995). Thus, the context of integrin-like protein compartmentalization within the cell may be a critical element related to a pathogenic phenotype. That is, while both *N. fowleri* and *N. lovaniensis* contained integrin-like proteins, only *N. fowleri* exhibited a co-localization with actin filaments at focal adhesion-like structures.

In order to garner additional insight into whether integrin-like proteins played a functionally relevant role in binding to the ECM, a peptide harbouring an integrin recognition site was used in binding inhibition experiments. The peptide selected contained an arginine-glycine-aspartic acid (RGD) motif, an acidic amino acid domain found in the integrin interaction site of many ECM proteins (Ruoslahti, 1996). However, the RGD peptide selected did not inhibit adhesion of *N. fowleri* to collagen I. One possible explanation for the lack of inhibition is that it has been reported that RGD-dependent attachment of cells to collagen I occurs when this protein is in the denatured state. Thus, in the native conformation of collagen I, the RGD motif may not be accessible to binding of integrins (Barczyk *et al.*, 2010). In this context, a prerequisite for RGD-mediated binding to collagen I would be a prior degradative step exerted on the part of the amoebae. That is, proteases secreted from *N. fowleri* could serve to 'unmask' RGD tripeptide motifs on the target collagen 1. An alternative possibility is that the 53 kDa and/or 70 kDa proteins expressed on *N. fowleri* may be linked to binding to collagen 1 in an RGD-independent fashion. Indeed, two-thirds of integrins have been found to bind ligands in an RGD-independent manner (Barczyk *et al.*, 2010). To explore further the possibility of an integrin-like protein on *N. fowleri* as linked to binding to the ECM, an anti-integrin monoclonal antibody that inhibits attachment of mammalian cells to collagen I, fibronectin and laminin-1 was used in binding inhibition studies. This antibody decreased attachment of *N. fowleri* to ECM components. When an irrelevant antibody, namely anti-KLH antibody, was used in experiments, a 10 % decreased level of binding to collagen I, fibronectin or laminin-1 was obtained. In contrast, when the anti-integrin monoclonal antibody was used, a 30–45 % decreased level of binding to the ECM components was observed. This greater than twofold difference in decreasing the level of binding to ECM proteins implies a specificity of action of the anti-integrin antibody.

Finally, the ability of *N. fowleri* versus *N. lovaniensis* to invade collagen I or Matrigel matrices was assessed. Fibronectin and laminin-1 were not used for the invasion studies, as these components do not allow for formation of a 3D matrix structure. Invasion assays have been used to measure the 'invasiveness' of tumour cells (Kleinman & Jacob, 2001) and *Acanthamoeba* spp. (Rocha-Azevedo *et al.*, 2009). Microscopic examination of tissue has demonstrated that *N. fowleri* passes through the nasal epithelium and then penetrates the basement membrane as it migrates to the brain (Jarolim *et al.*, 2000). In addition, electron microscopic analysis of infected mouse brain has revealed amoebae apparently engulfing collagen fibrils during the invasion process (Martinez *et al.*, 1973). In the present study, *N. fowleri* were shown to more readily penetrate collagen I and Matrigel constructs. This apparent enhanced capability to penetrate the ECM 3D composite could be due to enhanced motility on the part of the amoeba. For example, Thong & Ferrante (1986) compared the migration patterns of *Naegleria* species under agarose and reported that their locomotive ability correlated with pathogenic potential. In addition, *N. fowleri*, but not *N. lovaniensis*, has been shown to exhibit enhanced motility when placed in proximity to rat B103 neuroblastoma cells (Cline *et al.*, 1986), suggesting that *N. fowleri* selectively

'senses' neurotropic factors as compared with *N. lovaniensis* and other free-living amoebae that lack neuropathogenic potential. Alternatively, the focal adhesion-like protuberances may serve as anchor points to the ECM and, in that capacity, exert tensional forces (Hersen & Ladoux, 2011) that disrupt the ECM. In this context, disruption of ECM scaffolds through the mediation of tensional forces has been observed for fibroblasts (Grinnell, 2008). The extent to which proteases released from *N. fowleri* (Aldape *et al.*, 1994) or tensional forces created by the amoebae–matrix interaction are involved in invasion of the ECM scaffold remains to be elucidated. Finally, 'food-cups' on amoebae do not appear to play a vital role in invasion of the ECM, since SEM revealed their presence on both species. It has been proposed that these structures are used by amoebae to ingest bacteria in the environment (Marciano-Cabral & Cabral, 2007).

In summary, two free-living amoebae from the genus *Naegleria*, one a pathogen in humans and the other a non-pathogen in humans, were compared for their ability to attach to, and invade, ECM proteins *in vitro*. *N. fowleri* exhibited a higher level of attachment to ECM components than *N. lovaniensis*. A distinctive morphological feature of *N. fowleri* was that it displayed focal adhesion-like structures to which $\beta 1$ integrin-like molecules co-localized with actin filaments. The role of the focal-like adhesions in the ECM attachment process, the functional relevance of integrin-like molecules that co-localize with actin filaments in this process, and the linkage of amoebic attachment to the ECM in induction of a signal transductional cascade that promotes invasion of the ECM, are under investigation.

ACKNOWLEDGEMENTS

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