Disruption of MDCK cell tight junctions by the free-living amoeba Naegleria fowleri

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Naegleria fowleri is the aetiological agent of primary amoebic meningoencephalitis. This parasite invades its host by penetrating the olfactory mucosa. However, the mechanism of epithelium penetration is not well understood. In the present study, we evaluated the effect of N. fowleri trophozoites and the non-pathogenic Naegleria gruberi on Madin–Darby canine kidney (MDCK) tight junction proteins, including claudin-1, occludin and ZO-1, as well as on the actin cytoskeleton. Trophozoites from each of the free-living amoeba species were co-cultured with MDCK cells in a 1 : 1 ratio for 1, 3, 6 or 10 h. Light microscopy revealed that N. fowleri caused morphological changes as early as 3 h post-infection in an epithelial MDCK monolayer. Confocal microscopy analysis revealed that after 10 h of co-culture, N. fowleri trophozoites induced epithelial cell damage, which was characterized by changes in the actin apical ring and disruption of the ZO-1 and claudin-1 proteins but not occludin. Western blot assays revealed gradual degradation of ZO-1 and claudin-1 as early as 3 h post-infection. Likewise, there was a drop in transepithelial electrical resistance that resulted in increased epithelial permeability and facilitated the invasion of N. fowleri trophozoites by a paracellular route. In contrast, N. gruberi did not induce alterations in MDCK cells even at 10 h post-infection. Based on these results, we suggest that N. fowleri trophozoites disrupt epithelial monolayers, which could enable their penetration of the olfactory epithelium and subsequent invasion of the central nervous system.

INTRODUCTION

Primary amoebic meningoencephalitis (PAM) in humans is caused by the protozoan parasite Naegleria fowleri. PAM is an acute and rapidly fatal infection that is characterized by parasitic invasion of the central nervous system (CNS). The parasite gains access to the CNS by penetrating the olfactory neuroepithelium and migrating through olfactory nerves until it reaches the olfactory bulbs (Carter, 1970; Jaroli et al., 2002; Jarolim et al., 2000; Rojas-Hernández et al., 2004; Shibayama et al., 2003). Immunohistochemical studies of the early events of infection using a murine model have shown that the amoebae induce intense mucus secretion and an inflammatory reaction in the nasal cavity (Cervantes-Sandoval et al., 2008a; Rojas-Hernández et al., 2004). However, N. fowleri is able to evade host innate defence mechanisms by mucous degradation that occurs via a 37 kDa cysteine protease (Cervantes-Sandoval et al., 2008b), allowing the amoebae to adhere to the neuro-olfactory epithelium. A striking observation is that N. fowleri transits the epithelium (Cervantes-Sandoval et al., 2008a; Rojas-Hernández et al., 2004) by traversing cell junctions without causing any apparent damage.

Tight junctions (TJs) constitute one of the first barriers against invasion by various micro-organisms. The expression of tissue-specific transport proteins and channels is crucial for substance exchange between the internal and external cellular environment that occurs at TJs (Ohland & Macnaughton, 2010). The transmembrane proteins that constitute TJs are attached to the cytoskeleton, thereby...
linking one cell to another (Wit Jensen et al., 2000). TJ are composed of the transmembrane proteins claudin, occludin and junctional adhesion molecule (JAM) (Tsukita et al., 2001). Claudin and occludin span the plasma membrane four times; each protein contains two extracellular loops, one intracellular loop and two cytosolic termini. The extracellular loops of adjacent cells bind to each other and generate close membrane proximity (González-Mariscal et al., 2003; Turner, 2006). The carboxy termini of both occludin and claudin are associated with the guanylate kinase homologues ZO-1, ZO-2 and ZO-3.

Some micro-organisms, such as bacteria, viruses, nematodes, and even protozoa like Entamoeba histolytica and Acanthamoeba castellanii, are able to disassemble TJ proteins and thus invade the host and cause serious diseases (Förster, 2008; Khan, 2008; Leroy et al., 2000). E. histolytica degrades ZO-1 by a mechanism involving phosphorylation and dephosphorylation (Leroy et al., 2000; Que & Reed, 1997). Recently, it was reported that A. castellanii, a free-living amoeba, affects TJs at the blood–brain barrier (Khan, 2008). In contrast, there is no information regarding the penetration mechanism for N. fowleri into the olfactory epithelium. In the present study, we show the degradation of ZO-1 and claudin-1 in TJs and actin apical ring alteration in Madin–Darby canine kidney (MDCK) monolayers by N. fowleri trophozoites.

**METHODS**

### Amebic and cell cultures. The pathogenic strain N. fowleri (ATCC 30808) and the non-pathogenic strain Naegleria gruberi (kindly provided by Dr G. Vives Varela, CDC, Atlanta GA, USA) were used in all experiments. Trophozoites were axenically cultured in 2 % (w/v) bacto casitone medium supplemented with 10 % (v/v) fetal bovine serum (FBS; Equitech-bio) at 37 °C for N. fowleri and at room temperature for N. gruberi. Trophozoites were harvested during the exponential growth phase (48 h). MDCK cells were grown in minimal essential medium (MEM; Gibco Invitrogen) with 10 % (v/v) FBS (Equitech-bio) in a 5 % CO2 atmosphere at 37 °C.

### Interactions of MDCK cells and Naegleria strains. Trophozoites of N. fowleri and N. gruberi were removed from the culture flask surface by incubating in an ice bath for 10 min, centrifuging at 2100 g for 10 min, and washing twice with PBS (pH 7.2). Trophozoites of each strain were adjusted to 6 × 106 cells in 5 ml FBS-free MEM and added to confluent monolayers of MDCK cells (ratio 1:1). Conditioned medium (CM) was obtained as previously reported by Serrano-Luna et al. (2007). Co-cultures with N. fowleri trophozoites or CM were incubated at 37 °C in a 5 % CO2 atmosphere, and co-cultures with N. gruberi trophozoites or CM were incubated at room temperature for 1, 3, 6 or 10 h. Control MDCK cells were grown without amoebae for the same incubation times. The samples were prepared for light microscopy or fixed for confocal microscopy analysis.

### Transepithelial electrical resistance (TEER) in MDCK cells after co-incubation with free-living amoebae. TEER of cultured monolayers was measured directly using an epithelial voltmeter (EVOM). Briefly, 6 × 105 MDCK cells were co-incubated in a 1:1 ratio with each amoebic strain, which was seeded in 12 mm2 permeable transwell supports, containing the MDCK cell monolayer (0.4 μm pore size; Costar, Corning). The electrodes were cleaned with 70 % ethanol and rinsed with sterile PBS before measurements were taken. One electrode was immersed in the medium covering the MDCK monolayers, while the other electrode was immersed in the medium on the outside of the transwell. The resistance was registered in Ω cm−2. When the resistance was stable (approx. 400 Ω cm−2), the monolayers were washed with PBS and co-incubated with the amoebae in MEM. In order to avoid interference in TEER quantification by trophozoites, we removed them from cell monolayers by chilling in an ice bath for 20 min. Then TEER was quantified for periods of 1, 3, 6 or 10 h. The resistance data were obtained from three independent experiments. The results were compared with an experimental control (cells incubated without amoebae) and are reported as relative percentages. The data were analysed with the Systat software Sigma Plot 12 (http://www.sigmaplot.com).

### Confocal microscopy analysis of the MDCK cell TJs during coculture with N. fowleri or with N. gruberi. MDCK monolayers used for fluorescent staining were grown on coverslips pre-treated with 3 % Sylene (Alidrach) and 5 × 105 cells were incubated with N. fowleri or N. gruberi in a 1:1 cell ratio. After 1, 3, 6 or 10 h of incubation, MDCK cells were washed twice in cold PBS (4 °C) and fixed with 2 % paraformaldehyde in PBS for 20 min at room temperature. Samples were permeabilized with 0.2 % (w/v) Triton X-100 in PBS for 15 min at room temperature and washed three times with cold PBS. Coverslips were blocked with 1 % albumin for 1 h at room temperature. Cells were labelled with a polyclonal mouse anti-occludin antibody (1:200), rabbit anti-claudin-1 antibody (1:200) or rabbit anti-ZO-1 antibody (1:400) (Zymed Laboratories). Cells were incubated for 2 h at 37 °C with the primary antibody, washed three times with PBS, and incubated with the appropriate fluorescein-labelled secondary antibody [FITC-goat-anti-rabbit (1:25) for ZO-1 and claudin-1, FITC-goat-anti-mouse (1:25) for occludin; Santa Cruz Biotechnology]. Actin filaments were immunostained with rhodamine phalloidin (1:150) for 20 min at room temperature. Cell nuclei were stained with propidium iodide (0.001 %) for 5 min. Amoebae were immunolabelled with a polyclonal amoebic antibody (1:50) and a secondary antibody coupled to CY5 or FITC (1:50). Cells were incubated for 1 h at 37 °C and washed three times with cold PBS. Finally, the coverslips were mounted using Vectashield (Vector Laboratory). Images were obtained with an Olympus confocal microscope (model FV-500).

### MDCK cell lysates. MDCK cell lysates were obtained using lysis buffer [150 mM NaCl, 1 % (v/v) Triton X-100, 50 mM Trizma base] with a protease inhibitor cocktail. The protease inhibitors and their final concentrations were as follows: cysteine proteases, 10 mM p-hydroxymercuribenzoate; 5 mM N-ethylmaleimide and 10 μM trans-epoxyuccinyl-leucylamid[4-guanidine]butane (E-64) (Roche Applied Science); metalloproteases, 2 mM EDTA; serine proteases, 5 mM PMSF; and cysteine and serine proteases, 3 mM N-tosyl-L-lysine chloromethyl ketone (TLCK), 3 mM N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) and 5 μM iodoacetic acid (Sigma-Aldrich). Cell protein concentrations were quantified by the Bradford method.

### Detection of TJ degradation by Western blot assays. MDCK cells were recovered for electrophoresis and Western blot assays using lysis buffer with a protease inhibitor cocktail. Protein samples of MDCK cells were processed in sample buffer (Laemmli 5 x) with 5 % β-mercaptoethanol (Sigma). Protein was loaded at 40 μg per well. MDCK cell proteins were separated using 7.5 %, 10 %, 12 % or 15 % SDS-PAGE. Electrophoresis was performed at 4 °C in an ice bath at a constant voltage (100 V) for 1 h, and the proteins were transferred to PVDF membranes (Immobilon-P, Millipore). The transfer was performed at 4 °C for 45 min at 400 mA. PVDF membranes were blocked at room temperature with 5 % skim milk (Diffco) dissolved in PBS and incubated overnight at 4 °C. For protein immunodetection,
the membranes were incubated with a polyclonal mouse anti-
occludin antibody (1:200), anti-actin antibody (1:100), polyclonal
rabbit anti-claudin-1 antibody (1:200) or anti-ZO-1 antibody
(1:400) (Zymed) for 2 h at 37 °C. Membranes were washed three
times with 0.05% Tween 20 in PBS (PBS-T) and incubated with
secondary antibodies for 1 h at 37 °C. The secondary antibodies
were used at the following dilutions: goat-anti-mouse-IgG peroxidase-
conjugated, 1:500 for occludin and 1:2500 for actin; goat anti-rabbit
IgG peroxidase-conjugated, 1:1000 for ZO-1 and 1:500 for claudin-1
(Santa Cruz Biotechnology). Finally, the membranes were washed six
times in PBS-T and revealed with luminol kit reagent (Santa Cruz
Biotechnology) using photographic Kodak film. Densitometry
analysis was performed using the ImageJ program (http://rsb.info.
nih.gov/nih-image), and the results are expressed as relative optical
densities (RODs).

**Prevention of TJ damage by a cysteine protease inhibitor.**
Pathogenic trophozoites were adjusted to 6 × 10^6 cells in 5 ml FBS-
free bactocasitone medium and incubated with 2 μM E-64 for 45 min
(trophozoite viability was evaluated by trypan blue staining and
indicated that 99% of the trophozoites were viable). The trophozoites
were washed three times with PBS (pH 7.2) and added to confluent
monolayers of MDCK cells (ratio 1:1). Co-cultures were incubated at
37 °C in a 5% CO2 atmosphere for up to 10 h. Finally MDCK cells
were observed by light microscopy; duplicate samples were processed
to assess TJ proteins by Western blot. Densitometry analysis was
performed using ImageJ and results expressed as RODs. Additionally,
TEER analysis was performed.

**RESULTS**

**Changes in epithelial cell morphology induced by N. fowleri trophozoites**

*N. fowleri* and *N. gruberi* trophozoites were incubated with
MDCK cells for 1, 3, 6 or 10 h. Morphological analyses showed that *N. fowleri* adhered to the MDCK cells beginning after the first hour of incubation, and 3 h following the start
of co-cultivation, structural changes were observed in the
monolayer, which were characterized by monolayer discon-
tinuity (Fig. 1a). At 6 and 10 h post-infection, the integrity
of the monolayer showed significant areas of damage (Fig.
1b, c), which decreased the cell viability by about 25% at
10 h post-infection (data not shown). In contrast, *N. gruberi*
(a non-pathogenic strain) was unable to cause damage at
any time after its infection of the MDCK cell monolayer
(Fig. 1d). As a negative control, MDCK cells were cultured in
the absence of amoebae, and the control cells displayed
complete monolayer integrity (data not shown).

**N. fowleri disrupts the TEER of MDCK cells**

To characterize the morphological cell damage, the initial
functional damage was determined by measuring the TEER
of MDCK monolayers that were exposed to trophozoites.
*N. fowleri* or *N. gruberi* trophozoites were attached to the
apical side of the MDCK cell monolayers. *N. fowleri* caused
a 30% decrease in the TEER after the first hour of co-
culture. Moreover, the TEER further decreased until it
reached 40% of the initial value at 3 h post-infection, and
10% of the initial value at 10 h (Fig. 2). These data
correlate with those presented in Fig. 1, which illustrate the
discontinuity of the epithelial monolayer. In the case of the
*N. gruberi* strain, the TEERs were unaltered following all
incubation times, similar to the TEERs measured for
MDCK cells that were not exposed to free-living amoebae
(Fig. 2).

**Fig. 1.** Light microscopy analysis of MDCK cells co-cultured with
amoebic trophozoites. Co-culture of MDCK cells with *N. fowleri* at
(a) 3 h, (b) 6 h and (c) 10 h post-infection and with *N. gruberi* at
10 h post-infection (d). Arrows highlight areas of monolayer
damage. Images were obtained with an inverted Nikon microscope
(TMS-F) at ×40.

**Fig. 2.** TEER in MDCK cells after co-culture with free-living amoebae. *N. fowleri* (●) abolished TEER, and *N. gruberi* (○) had
no effect. The percentage of TEER is compared with MDCK control
values (▼). The data are the means ± SEM of three independent experiments.
**N. fowleri* trophozoites induce actin cytoskeleton alterations in MDCK cells**

To further understand the interaction of *N. fowleri* trophozoites with intercellular adhesion proteins in MDCK cells, actin apical ring integrity was investigated. Because actin fibres form a ring that circumscribes the plasma membrane and defines the apical pole, MDCK F-actin was detected with rhodamine phalloidin during the interaction of MDCK cells with *N. fowleri* or *N. gruberi* free-living amoebae. At 3 h post-infection, the F-actin cytoskeleton of MDCK monolayers that were exposed to *N. fowleri* trophozoites displayed an altered morphology, which included actin reorganization and apical ring disassembly into individual actin stress fibres (Fig. 3b). At later times post-infection, actin disruption was more evident, including a loss of continuity in the actin apical ring (Fig. 3b–d) and the presence of F-actin tufts at 6 and 10 h post-infection (Fig. 3c, d). Interestingly, *N. gruberi* trophozoites were unable to alter the actin cytoskeleton of MDCK cells at any time following infection (Fig. 3e–h). MDCK cells cultured in the absence of trophozoites showed normal actin cytoskeleton distribution and organization (Fig. 3i–l). Interestingly, confocal XZ stacks of actin organization showed that treated cells incubated with *N. fowleri* for 1 h preserved their actin apical ring organization (Fig. 3m) like the control of untreated cells at 10 h (data not shown); thereafter, *N. fowleri* trophozoites caused disorganization of this actin apical ring. The effect on actin organization was time-dependent (Fig. 3n–p); at 10 h the actin apical ring was completely disorganized and processes of cell sloughing were observed (Fig. 3p). Notably, the amoebic trophozoites from both strains exhibited a tropism for cell junction regions (Fig. 3a–h).

**N. fowleri** trophozoites induce the rearrangement of TJ proteins

The tropism of trophozoites to the intercellular boundaries and the disruption of the actin apical ring led us to further investigate the disruption of the TJ proteins. Because the apical ring and the TJs are located at the same plane in cells, claudin, occludin and ZO-1 were examined during trophozoite–MDCK cell interactions. The interaction of *N. fowleri* trophozoites with MDCK cells decreased the level of immunolabelling of ZO-1 and claudin-1, and led to a partial decrease in the immunolabelling of occludin. The decrease in ZO-1 and claudin-1 was time-dependent (Fig. 4a–f). The alteration in ZO-1 was most evident at 6 and 10 h post-infection, at which point the label was lost in...
some cells (Fig. 4b, c). Claudin-1 behaved similarly to ZO-1, with diffuse and discontinuous immunolabelling mainly at 6 and 10 h post-infection (Fig. 4e–f). In contrast, the changes in occludin levels were less evident than those in claudin-1 and ZO-1 at any time that was examined (Fig. 4g–i), with the most obvious changes occurring at 10 h post-infection. Co-cultures of non-pathogenic N. gruberi trophozoites with MDCK cells showed no alterations in the pattern of immunolabelling of ZO-1, claudin-1 or occludin at any time post-infection (Fig. 5a, c, e). Finally, as expected for the negative control cells, MDCK cells that were cultured in the absence of amoebae displayed constant protein levels throughout the cell culture period (Fig. 5b, d, f).

**Degradation of TJ proteins by N. fowleri trophozoites**

To analyse the possible degradation of MDCK TJ proteins during co-culture with N. fowleri or N. gruberi trophozoites for 1, 3, 6 or 10 h, we removed the trophozoites at each post-infection time and processed the MDCK monolayers to obtain crude cell extracts for analysis by SDS-PAGE at different acrylamide concentrations. Immunoblotting was performed on PVDF membranes with antibodies against actin, ZO-1, claudin-1 and occludin. We found that co-culture of MDCK cells with N. fowleri resulted in the degradation of ZO-1 and claudin-1, which was evident at 3 h post-infection and was time dependent (Fig. 6a). There was no significant degradation of occludin at any time post-infection (Fig. 6a) \( (P>0.05) \). In contrast, co-culturing the non-pathogenic N. gruberi strain with MDCK cells did not lead to the degradation of ZO-1, claudin-1 or occludin (Fig. 6a). We also assessed the integrity of actin in response to trophozoite co-culture. Actin was not degraded after any length of time of co-culture with N. fowleri or N. gruberi (Fig. 6a). These differences in proteolysis were evident when protein levels of each TJ protein were analysed with ImageJ and expressed as relative optical densities (RODs) (Fig. 6b).

**Cysteine protease inhibition with E-64 prevents MDCK TJ damage**

To explore the participation of the proteases secreted by N. fowleri (Serrano-Luna et al., 2007) in the interaction of this amoeba with MDCK cells, these cells were incubated with conditioned medium from N. fowleri or N. gruberi at different times. Only conditioned medium from N. fowleri was able to cause cell damage, as observed when living trophozoites were used. The highest effect was detected at 10 h of incubation (Fig. 7a) and this effect was not detected when conditioned medium from N. gruberi was used (Fig. 7b). In order to know if the proteases secreted by N. fowleri are implicated in this cell damage, the effect of a permeable cysteine protease inhibitor on TJ damage was determined. The cysteine protease inhibitor E-64 was used to pre-treat N. fowleri trophozoites for 45 min, followed by incubation of the trophozoites with MDCK cells for up to 10 h. Using light microscopy, we observed that the E-64 inhibitor per
**DISCUSSION**

*Naegleria fowleri* is the causal agent of PAM, and the acquisition of this entity is commonly associated with a recent history of aquatic activity by the patient (Schuster & Visvesvara, 2004). The route of invasion of *N. fowleri* has been analysed by histopathological studies using a mouse model (Cartier, 1970; Cervantes-Sandoval et al., 2008a; Jaroli et al., 2002). These studies revealed that the amoeba infects the host by first penetrating the olfactory mucosa and subsequently crossing the cribriform plate of the ethmoid bone, eventually reaching the olfactory bulbs of the CNS. In the early stages of infection, *N. fowleri* trophozoites are embedded in a large amount of mucus and surrounded by an inflammatory infiltrate composed mainly of neutrophils (Cervantes-Sandoval et al., 2008a; Rojas-Hernández et al., 2004). However, *N. fowleri* is able to degrade the mucus by secreting a 37 kDa cysteine protease (Cervantes-Sandoval et al., 2008b), which most likely allows the amoeba to evade the mucus and adhere to the olfactory epithelium (Cervantes-Sandoval et al., 2008a). For many micro-organisms, adherence is one of the most important features observed during the early invasion of different tissues or organs (Kucknoor et al., 2005; McCoy et al., 1994; Panjwani, 2010). *N. fowleri* is known to adhere to different cell types in a process that involves glycoconjugates as well as extracellular matrix proteins, such as fibronectin and collagen (Cervantes-Sandoval et al., 2010; Han et al., 2004; Shibayama et al., 2003). During the initial adherence step, *N. fowleri* may activate intracellular signals that could be important for traversing epithelial cells. In the present study, we focused our analysis on the amoeba–epithelium interaction to understand how this amoeba may cross the epithelium before invading the CNS. In a previous paper, we reported that *N. fowleri* trophozoites are capable of migrating through the neuroepithelium without producing any apparent damage, suggesting that this migration could occur via a paracellular route (Cervantes-Sandoval et al., 2008a). During evolution, pathogens acquire or adapt different mechanisms to intrude or penetrate various defence mechanisms of the host, such as mucus, inflammatory cells and epithelial barriers. At the cellular level, one of the strategies of pathogenic protozoa is to traverse the epithelium using a paracellular route by disrupting TJJs. Some parasites, such as *Giardia intestinalis* and *Trichomonas vaginalis*, do not invade host cells but rather decrease the cellular permeability of CaCo-2 epithelial cells. This effect was determined by examining TEER upon modifying the junctional complex in epithelial cells because these parasites alter the distribution of the ZO-1 and ZO-2 proteins (da Costa et al., 2005; Maia-Brigagão et al., 2012). In contrast, coculture of *E. histolytica* with an enteric T-84 cell line decreases the TEER by disrupting the TJ proteins via the dephosphorylation and degradation of ZO-1 (Leroy et al., 2000). More recently, Khan & Siddiqui (2009) reported that *A. castellanii*, a pathogenic free-living amoeba, releases secretory products into the culture medium, which results in the expression of zonula occludens (ZO-1) and ZO-2, which are important for the integrity of the epithelial barrier.

![Figure 5](http://mic.sgmjournals.org)  
**Fig. 5.** *N. gruberi* trophozoites are unable to damage MDCK cell TJ proteins. Analysis of TJ protein levels in MDCK cells infected with *N. gruberi* trophozoites for 10 h infection (a, c, e). As a control, MDCK cells were cultured without amoebae for 10 h (b, d, f). ZO-1 (a, b), claudin-1 (c, d) and occludin (e, f) were immunolabelled with FITC-conjugated antibodies. Cell nuclei were labelled with propidium iodide. Images were obtained via confocal microscopy (Olympus FV-500) at ×60. Scale bars, 50 μm.

se did not cause cell damage (Fig. 7d) and the cells resembled the untreated cells (Fig. 7c). In contrast, E-64 was able to block cell damage induced by *N. fowleri* trophozoites (Fig. 7f). The absence of cell damage correlated with a lack of proteolysis of TJ proteins, as Western blot analysis demonstrated no decrease in ZO-1, claudin-1 or occludin levels following the co-culture of MDCK cells with E-64 pre-treated *N. fowleri* trophozoites (Fig. 7g). E-64 also caused inhibition of the TEER decrement induced by *N. fowleri* trophozoites (Fig. 7j). As expected, MDCK cells without E-64 pre-treatment showed cell damage and TJ disruption at 10 h of interaction with the amoebae (Fig. 7e, g). The differences in proteolysis for each TJ protein were quantified by measuring the protein levels with ImageJ (Fig. 7f). Previously, our group showed that cysteine proteases are the main group secreted by *N. fowleri* and lesser quantities of serine proteases are found (Serrano-Luna et al., 2007). Consistent with this observation, we found that aprotinin, a non-toxic serine protease inhibitor, did not protect against TJ disruption induced by *N. fowleri* (data not shown).
in the degradation of ZO-1 and occludin in human brain microvascular endothelial cells (HBMEC) (Khan & Siddiqui, 2009). In the present study, we analysed the interaction of epithelial MDCK cells with N. fowleri and N. gruberi trophozoites. Similar to E. histolytica trophozoites, we found that N. fowleri causes an important decrease in the TEER, but this decrease occurs at a slower rate than that caused by E. histolytica (Leroy et al., 2000). In addition to the degradation of ZO-1, we also observed claudin-1 degradation. However, as reported for A. castellanii trophozoites, occludin was not degraded (Khan & Siddiqui, 2009). N. fowleri trophozoites are known to produce cysteine and serine proteases that are considered to be important virulence factors and these proteases could participate in TJ disruption (Aldape et al., 1994; Ferrante et al., 1988; Serrano-Luna et al., 2007). To test this possibility, we pre-treated N. fowleri trophozoites with E-64, a cysteine protease inhibitor, and found that E-64 abrogated MDCK TJ degradation, but this inhibition was not observed when aprotinin was used, a serine protease inhibitor. Similarly, Lauwaet et al. (2004) revealed that TPCK and TLCK, two cysteine and serine protease inhibitors, are able to inhibit E. histolytica cysteine proteases and prevent the proteolysis of ZO-1, ZO-2 and villin in Caco-2 cells (Lauwaet et al., 2004). Nikolskaia et al. (2006) found that the crossing of HBMEC by Trypanosoma brucei gambiense was abrogated by N-methylpiperazine-urea-Phe-homophenylalanine-vinyl sulfone-benzene (K11777), an irreversible inhibitor of cathepsin L-like cysteine proteases (Nikolskaia et al., 2006). Finally, Sissons et al. (2006) used an isolate of Acanthamoeba sp. to evaluate the ability of PMSF (a serine protease inhibitor) to inhibit TJ degradation in HBMEC cells (Sissons et al., 2006). In other microorganisms, such as Bacteroides fragilis and Helicobacter pylori, it has been shown that the release of zinc-dependent metalloproteases causes the destabilization of TJs (Wu et al., 1998). Here we report, we believe for the first time, the disruption and degradation by which N. fowleri alters TJ proteins. This phenomenon may occur during the process of tissue invasion, both at the olfactory neuroepithelium and at the blood–brain barrier. However, it will be necessary to perform in vivo assays to validate the hypothesis that amoebic migration occurs through a paracellular route to provide further evidence for this mechanism of N. fowleri pathogenicity. Importantly, the non-pathogenic strain N. gruberi did not disrupt MDCK cell TJs, although it is known that this strain also releases some cysteine proteases as well as other types of proteases (Serrano-Luna et al., 2007). Recently, it has been reported that there are differences

**Fig. 6.** Degradation of TJ proteins by N. fowleri trophozoites. (a) Western blot analysis of the MDCK proteins ZO-1, claudin-1, occludin and actin after co-culturing with N. fowleri or N. gruberi for different times. Controls were MDCK cells that were cultured without amoebae for different times. (b) Densitometric analyses were performed with GraphPad Prism. Control, black bars; N. fowleri or N. gruberi co-culture samples, white bars. All bars show the mean ± SEM of three independent assays (*P < 0.05).
between pathogenic and non-pathogenic strains, including the differential expression of recognition molecules important for the adhesion and invasion steps of infection, such as lectins and extracellular matrix proteins (Jamerson et al., 2012). These and other differences between \textit{N. fowleri} and \textit{N. gruberi} amoebae may explain the lack of virulence of \textit{N. gruberi}. In summary, secreted cysteine proteases from adhered \textit{N. fowleri} trophozoites are able to degrade ZO-1 and claudin-1 but not occludin; actin is not degraded, but the actin apical ring is disorganized and long exposure (10 h) can lead to cell sloughing. It is interesting to note that any of these degraded components causes TJ disruption. However, occludin can be endocytosed since it is not degraded by \textit{N. fowleri}. Occludin could be cleaved in its long carboxyl tail ending in a coiled-coil domain made of three alpha helices.

![Fig. 7. E-64 prevents the degradation of MDCK TJ proteins during co-culture with \textit{N. fowleri}. (a–f) Light microscopy of MDCK epithelial cells, co-cultured (a) with conditioned medium of \textit{N. fowleri}; (b) with conditioned medium of \textit{N. gruberi}; (c) in MEM; (d) in MEM with E-64; (e) with \textit{N. fowleri} trophozoites; (f) with \textit{N. fowleri} trophozoites pre-treated with E-64 (2 \textmu M) for 45 min. All samples were cultured for 10 h. Images were obtained with an inverted Nikon microscope (TMS-F) at ×10 (a, b, c, d, f) or ×20 (e). (g) Western blots of ZO-1, claudin-1, occludin and actin proteins, after co-culturing with \textit{N. fowleri} pre-treated with E-64. (h) Densitometric analyses were performed with GraphPad Prism. \textit{N. fowleri} in the presence of E-64, white bars; control MDCK cells with E-64, light grey bars; \textit{N. fowleri} without E-64, dark grey bars. All bars show the mean ± SEM of three independent assays (*\textit{P} < 0.05). (i) TEER assays. MDCK cells were co-incubated with \textit{N. fowleri} (▼) or \textit{N. fowleri} pre-treated with E-64 (▪). The percentage of TEER is compared with MDCK control values (●). The data were analysed with the Systat software Sigma Plot 12. All data are the mean ± SEM of three independent assays (\textit{P} < 0.05).
helices. This coiled-coil interacts with ZO-1. In fact in the absence of proteases, using Lantrunculin A (a drug) it is possible to disassemble the actin apical ring, which leads to TJ disassembly and endocytosis of the transmembrane components such as claudins and occludin, without proteolysis (Shen & Turner, 2005). Thus, various events indicate that N. fowleri can use the paracellular route to cause PAM: (1) the amoeba has been seen invading the olfactory epithelium without evidence of cellular damage or ulceration (in animal models); (2) as shown here, using cultured cells, N. fowleri causes loss of cell viability of about 25 % after 10 h of interaction, which is not a really strong cytolytic effect; (3) amoebal conditioned medium is able to cause TJ protein disruption without destruction of MDCK cells. This TJ disruption led to cell sloughing, whereby the amoebae can move to invade the neuro-olfactory epithelium.

In conclusion, our study demonstrates that pathogenic N. fowleri trophozoites can disrupt MDCK TJs by degrading ZO-1 and claudin-1 using amoebic cysteine proteases. These results suggest that N. fowleri could use this mechanism to invade the neuro-olfactory epithelial cells and/or the blood–brain barrier of the host.

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