Mucins in the host defence against *Naegleria fowleri* and mucinolytic activity as a possible means of evasion

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Naegleria fowleri is the aetiological agent of primary amoebic meningoencephalitis (PAM). This parasite invades its host by penetrating the olfactory mucosa. During the initial stages of infection, the host response is initiated by the secretion of mucus that traps the trophozoites. Despite this response, some trophozoites are able to reach, adhere to and penetrate the epithelium. In the present work, we evaluated the effect of mucins on amoebic adherence and cytotoxicity to Madin–Darby canine kidney (MDCK) cells and the MUC5AC-inducing cell line NCI-H292. We showed that mucins inhibited the adhesion of amoebae to both cell lines; however, this inhibition was overcome in a time-dependent manner. *N. fowleri* re-established the capacity to adhere faster than *N. gruberi*. Moreover, mucins reduced the cytotoxicity to target cells and the progression of the illness in mice. In addition, we demonstrated mucinolytic activity in both *Naegleria* strains and identified a 37 kDa protein with mucinolytic activity. The activity of this protein was inhibited by cysteine protease inhibitors. Based on these results, we suggest that mucus, including its major mucin component, may act as an effective protective barrier that prevents most cases of PAM; however, when the number of amoebae is sufficient to overwhelm the innate immune response, the parasites may evade the mucus by degrading mucins via a proteolytic mechanism.

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INTRODUCTION

Naegleria fowleri is an amphotrophic amoeboflagellate and the aetiological agent for primary amoebic meningoencephalitis (PAM), an acute and rapidly fatal central nervous system (CNS) disease. It has been demonstrated that this protozoan gains access to the CNS by penetrating the olfactory neuroepithelium and invading the olfactory bulbs (Carter, 1970; Jarolim *et al.*, 2000, 2002; Rojas-Hernández *et al.*, 2004). Once in the CNS, the amoebae induce a strong inflammatory response with lysis of leukocytes that contributes to tissue damage (Cervantes-Sandoval *et al.*, 2008). Immunohistochemical studies of the early events of infection using a murine model have shown that once the

amoebae reach the olfactory cavity, an intense secretion of mucus is produced by the host (Rojas-Hernández *et al.*, 2004). This mucus secretion envelops the *N. fowleri* trophozoites and then an intense inflammatory reaction is generated, composed primarily of neutrophils. This is the first host innate immune response that attempts to eliminate the foreign agent. It has been reported that other protozoan parasites that infect their host by invading mucosal tissues produce lytic enzymes that degrade mucus glycoproteins (Lehker & Sweeney, 1999; Moncada *et al.*, 2003a, 2005). This degradation of mucins is essential for parasite evasion of the host response and to gain final access to target tissues. On the other hand, it has been shown that *N. fowleri* has a variety of virulence factors that may help the parasite to evade the host immune response and finally invade the tissue. These factors are mainly cysteine proteases (Aldape *et al.*, 1994; Mat Amin, 2004; Serrano-Luna *et al.*, 2007), phospholipases (Fulford & Marciano-Cabral, 1986; Barbour & Marciano-Cabral,

Abbreviations: BSM, bovine submaxillary mucin; MDCK, Madin–Darby canine kidney; MTT, thiazolyl blue tetrazolium bromide; PAM, primary amoebic meningoencephalitis; pHMB, *p*-hydroxymercuribenzoate; p.i., post inoculation; PMA, phorbol 12-myristate 13-acetate; PSM, porcine stomach mucin.

2001), pore-forming proteins (Young & Lowrey, 1989; Herbst *et al.*, 2002, 2004), and membrane capping and shedding formation (Shibayama *et al.*, 2003). In the current study, we evaluated the role of mucins as a natural immune response, and the role of mucinolytic activity in trophozoites of the pathogen *N. fowleri* and the non-pathogen *N. gruberi* as an evasive mechanism. We showed that mucins inhibit the adhesion of parasites to culture plates, and to epithelial, Madin–Darby canine kidney (MDCK) and the mucin-inducing cells, NCI-H292. This inhibition of adhesion by mucins was overcome by parasites in a time-dependent manner. Moreover, mucins delayed, but could not arrest, the cytotoxic effect of *N. fowleri* upon epithelial cells, as well as retarding the progression of illness in mice. Hence, we demonstrated the presence of mucinolytic activity in *Naegleria* spp. crude extracts and in live trophozoites. Finally, we identified a 37 kDa protein with mucinolytic activity, and this enzymic activity was inhibited by cysteine protease-specific inhibitors. These data suggest that mucinolytic activity, particularly the 37 kDa cysteine protease in *N. fowleri*, has an important role in mucin degradation and in the evasion of this host innate response.

METHODS

Amoebic and cell cultures. The pathogenic strain *N. fowleri* (ATCC 30808) and the non-pathogenic strain *N. gruberi* (kindly provided by Dr G. Visvesvara, CDC, Atlanta, GA, USA) were used in all experiments. Trophozoites were axenically cultured in 2% (w/v) bactocastone 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 (Hyclone) in a 5% CO₂ atmosphere. For experiments involving adhesion and cytotoxicity, cells were grown in 24-well culture plates (Costar) in 1 ml MEM to 90% confluence, and were serum-starved when needed for experiments.

NCI-H292 cells were grown in 1640 RPMI medium with 10% (v/v) FBS (Hyclone) and HEPES (25 mM) at 37 °C, in a 5% CO₂ atmosphere. After reaching semi-confluence (80%), they were serum-starved for 24 h to maintain low basal levels of mucin expression. After 24 h of serum starvation, cells were treated with phorbol 12-myristate 13-acetate (PMA; 10 ng ml⁻¹) for 1 h, for MUC5AC mucin induction, the most abundant airway mucin in humans. Then, cells were washed three times with serum-free medium and cultured for an additional 24 h. Finally, cell-culture supernatants and cell lysates were collected to measure MUC5AC protein and mRNA expression.

Determination of MUC5AC expression in NCI-H292 cells. mRNA and protein MUC5AC were determined in PMA-stimulated and non-stimulated cells. For RNA expression, after stimuli, total RNA was isolated using the QIAamp RNA Blood kit (Qiagen) and mRNA was amplified using the OneStep RT-PCR kit according to the manufacturer's instructions (Qiagen). The MUC5AC cDNA was amplified using the sense primer 5'-CAC CAA ATA CGC CAA CAA GAC-3' and the antisense primer 5'-CAG GGC CAC GCA GCC AGA GAA-3' reported by Shimizu *et al.* (2003). The glyceraldehyde dehydrogenase was amplified using the sense primer 5'-CCA CCC

ATG GCA AAT TCC ATG GCA-3' and the antisense primer 5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'.

MUC5AC protein was determined by immunocytochemical analysis and dot blotting. For immunocytochemistry, stimulated and non-stimulated cells were fixed with 2% (w/v) paraformaldehyde and stained with mouse mAb to MUC5AC (clone 1-13M1, Abcam) and FITC-labelled secondary antibody to mouse IgG (Zymed). Nuclei were stained with propidium iodide. Samples were examined under a confocal microscope (Olympus IX71).

NCI-H292 cell lysates were obtained using a lysis buffer [150 mM NaCl, 1% (v/v) Triton X-100, 50 mM Trizma]. For dot blotting, 2 µl total crude cell lysate or conditioned cultured medium was absorbed on a nitrocellulose membrane and blocked with 5% skimmed milk for 1 h. Then, the membrane was washed four times with PBS–Tween (0.05%, v/v) and incubated with the same mAb to MUC5AC for 2 h. The membrane was washed four times and incubated with an HRP-labelled secondary antibody to mouse IgG. Finally, the membrane was washed six times and revealed with luminol kit reagent (Santa Cruz Biotechnology). As a control, RPMI fresh culture medium or lysis buffer was tested for the presence of MUC5AC.

Experimental animals. Four-week-old male BALB/c mice were lightly anaesthetized in an ether chamber and inoculated by the nasal route with 20 µl fresh amoebic culture medium without FBS containing 2.5×10^4 *N. fowleri* trophozoites. After different post-inoculation (p.i.) intervals (1, 6 and 12 h), the animals were euthanized by an overdose of sodium pentobarbital. The brains were fixed by vascular perfusion with a 4% (w/v) solution of paraformaldehyde diluted in PBS (pH 7.2). The skulls were then carefully dissected and decalcified in 8% (w/v) EDTA–PBS for 5 days with daily changes of EDTA and constant agitation. After decalcification, the brains were processed using standard techniques and embedded in paraffin. Sections, 7 µm in thickness, were stained by an immunohistochemical technique using an in-house rabbit polyclonal anti-*N. fowleri* antibody [diluted 1:50 in PBS–Tween (0.05%, v/v)] for 1 h at room temperature. After washing the slides, a secondary antibody, anti-rabbit IgG labelled with peroxidase (diluted 1:200; Sigma-Aldrich), was used. Finally, the slides were washed with PBS–Tween and a diaminobenzidine kit (Pierce) was used for colour development.

The protocols for animal care were previously approved by the Institutional Committee (IACUC; ID number 244/05). The institution fulfils all the technical specifications for the production, care and use of laboratory animals, and is certified by a national law (NOM-062-ZOO-1999). All mice were handled according to the guidelines of the 2000 AVMA Panel of Euthanasia.

Effects of mucin on amoebic adhesion to culture plates and target cells. To determine the binding of both amoebic strains to mucin, 24-well culture plates were coated at 37 °C until dry with 300 µl of a 100 µg ml⁻¹ solution of bovine submaxillary mucin (BSM; Worthington) or a 100 µg ml⁻¹ solution of porcine stomach mucin (PSM; Sigma-Aldrich). Then, the plates were washed twice with PBS (pH 7.2) and 2.5×10^4 trophozoites were added to each well and incubated for different lengths of time (5, 15 and 30 min, and 1, 2 and 4 h) in 1 ml MEM. After the incubation period, the non-adherent trophozoites were collected by aspiration in an Eppendorf tube and quantified in a Neubauer chamber. Finally, the percentages of adhesion were determined and the results were expressed as the means $\pm$ SD of three replicates of at least three different experiments.

To determine the effect of mucins on trophozoite adhesion to MDCK cells, 2.5×10^4 trophozoites were incubated with an MDCK cell monolayer in 1 ml MEM without FBS containing different concentrations of BSM or PSM (0, 0.01, 0.1 and 1 mg ml⁻¹) in a 24-well

culture plate for 1 h or for different lengths of time (5, 15 and 30 min, and 1, 2 and 4 h). At the end of the incubation period, the non-adherent trophozoites were collected in an Eppendorf tube by aspiration and the adhesion was quantified as in the previous experiment.

To determine the amoebic adhesion to NCI-H292 mucin-inducing cells, *Naegleria* spp. trophozoites were added to PMA-stimulated cells (high mucin expression) or to non-stimulated cells (low basal mucin expression) at different time points and the adhesion was measured as described above.

Effect of mucins on the cytotoxicity of *Naegleria* spp. towards cultured cells. MDCK cell monolayers grown in 24-well culture plates were incubated with 5×10^4 of live trophozoites of both *Naegleria* strains for 12 h in MEM without FBS and supplemented with different concentrations of BSM or PSM (0, 0.1, 0.5, 1 and 2 mg ml⁻¹). To detach the adhered trophozoites after the incubation period, the plates were chilled in an ice bath for 10 min, and washed three times with ice-cold PBS. Then, 200 µl thiazolyl blue tetrazolium bromide (MTT; 0.5 mg ml⁻¹) diluted in MEM was added and incubated for 30 min at 37 °C. After incubation, the MTT solution was removed and the cells were washed twice with PBS. For colour exclusion, 220 µl DMSO was added to each well and incubated for 5 min at room temperature. Finally, A_{540} was determined. The percentage damage was calculated, with the absorbance of the cell monolayer incubated without trophozoites taken as zero cytotoxicity. In addition, the amoebic cytotoxicity to PMA-stimulated or non-stimulated NCI-H292 cells after 12 and 18 h of incubation was determined. Results were reported as the means $\pm$ SD of the percentage cytotoxicity of three replicates of at least three different experiments.

Effect of mucins in PAM pathogenesis *in vivo*. To evaluate the effect of mucins on the pathogenesis of PAM *in vivo*, 4-week-old BALB/c mice were lightly anaesthetized in a chamber with ether, then infection was induced in the mice by inoculation of a lethal dose of live trophozoites (2.5×10^4) in 20 µl fresh bactocasitone medium alone or supplemented with different concentrations of BSM or PSM (1, 2 and 5 mg ml⁻¹). The survival curves were then determined and the significance of the differences was calculated using the log-rank test (Bland & Altman, 2004).

Determination of mucinolytic activity and mucin substrate gel electrophoresis. Total crude amoebic extracts and conditioned medium (CM) of both amoebic strains were prepared as previously described (Serrano-Luna *et al.*, 2007). The CM was concentrated by two consecutive precipitations using ammonium sulfate. In brief, a cold saturated solution of ammonium sulfate was slowly added with constant agitation to 10 ml CM in an ice bath to reach a final concentration of 70 % (v/v). The solution was then centrifuged at 2000 g at 4 °C for 30 min. The pellet was resuspended in 10 ml cold PBS and the precipitation was repeated again. Finally, the pellet was reconstituted in 2 ml PBS and dialysed against PBS for salt elimination.

For the determination of mucinolytic activity, 200 µl PSM or BSM (2 mg ml⁻¹) was co-incubated at 37 °C with either 20 µl total crude extract (4 mg ml⁻¹) or 100 µl concentrated CM. Live trophozoites (1×10^6) were also incubated in 1 ml serum-free bactocasitone medium supplemented with BSM or PSM (2 mg ml⁻¹). Twenty-microlitre samples of each experiment were withdrawn at different post-incubation periods (0.5, 1, 3, 6, 12 and 24 h), mixed with sample buffer, and electrophoresed in SDS-PAGE at 7.5 %. Finally, gels were stained with Schiff's reagent (Sigma-Aldrich). The amoebic extract or CM components were unstained in the control gels when this technique was used (data not shown). The optical density of the bands was analysed in three replicates using the NIH image software

ImageJ (<http://rsb.info.nih.gov/ni-image>) and plotted. The degradation of MUC5AC was determined as follows: 200 µl PMA-stimulated NCI-H292 CM tested for the presence of MUC5AC was co-incubated with 20 µl amoebic total crude extract (4 mg ml⁻¹) or with 100 µl concentrated amoebic CM at 37 °C. Live trophozoites (1×10^6) were also incubated in 1 ml NCI-H292 CM. Two-microlitre samples from each experiment were withdrawn at different post-incubation periods (0.5, 1, 3, 6, 12 and 24 h) and dot-blotted as described above to detect the presence of residual MUC5AC.

Amoebic mucinases were determined in both strains by performing electrophoresis of crude amoebic extracts and CM in 15 % SDS-PAGE co-polymerized with 0.1 % (w/v) BSM or PSM as substrate (Lehker & Sweeney, 1999). Thirty micrograms of crude extract protein and 25 µl concentrated CM of *N. fowleri* or *N. gruberi* were loaded per well. Electrophoresis was performed at 4 °C in an ice bath and at a constant voltage (100 V) for 2 h; the gels were washed twice for 30 min with orbital agitation in 2.5 % (v/v) Triton X-100 (Sigma-Aldrich). The gels were then incubated overnight with one of the following buffer solutions: 100 mM acetic acid (pH 3.0), 100 mM sodium acetate (pH 5.0), 100 mM Tris-OH (pH 7.0) and 100 mM glycine (pH 9.0) at 35 °C. All buffers contained 2 mM CaCl₂. Finally, gels were stained with Schiff's reagent.

Different inhibitors of the four groups of proteases were tested for inhibition of mucinolytic activity. In the determination of mucinolytic activity, inhibitors were added to total crude extracts or CM during co-incubation with mucin solutions, or were pre-incubated for 1 h prior to substrate electrophoresis. The inhibitors and final concentrations used were as follows: for aspartic proteases, 1 µM pepstatin A; for cysteine proteases, 10 mM *p*-hydroxymercuribenzoate (pHMB) and 10 µM *trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64); for metalloproteases, 2 mM EDTA; for serine proteases, 5 mM PMSF and 6 µM aprotinin (Sigma-Aldrich).

RESULTS

N. fowleri induces mucus production in mice

To determine the induction of mucus secretion in mice by *N. fowleri*, histological analyses of the nasal cavity infected with live trophozoites were performed. It was shown that the host reacted as early as 1 h p.i. with a strong production and secretion of mucus, as reported elsewhere (Rojas-Hernández *et al.*, 2004). Before contacting the epithelial cells, trophozoites were intermixed with mucus in the nasal cavity (Fig. 1a). Six hours p.i., amoebae were seen again covered by the mucus secretion and surrounded by a strong inflammatory reaction, composed mainly of neutrophils (Fig. 1b). At 12 h p.i., amoebae had adhered to or were penetrating the neuroepithelium (Fig. 1c, d). Control animals inoculated with fresh culture medium did not show any changes of the olfactory neuroepithelium (data not shown). These results suggest that amoebic mechanisms to evade the mucus and the primary inflammatory response could be important in the pathogenesis of PAM.

Mucins inhibit amoebic adhesion to culture plates and MDCK cells

To test the role of mucins in avoiding the adhesion of *N. fowleri* and *N. gruberi* to substrate or to epithelial target

Fig. 1. Immunohistochemistry of nasal cavity of mice infected with live *N. fowleri*. (a) One hour after intranasal inoculation, only a few trophozoites (arrows) were observed in the nasal lumen enveloped in mucus secretion (m) in proximity to the olfactory neuroepithelium (oe); magnification $\times 20$. (b) Six hours p.i., live trophozoites (arrow) were observed within the mucus secretions and surrounded by inflammatory cells (arrow heads); magnification $\times 20$. (c) A single trophozoite (arrow) was observed adhered to the apical zone of the olfactory epithelium (oe); magnification $\times 40$. (d) Twelve hours p.i., a trophozoite was observed penetrating the epithelium (oe); magnification $\times 40$. Immunoperoxidase reaction using an anti-*N. fowleri* antibody. Bars, (a, b), $70\ \mu\text{m}$; (c, d), $35\ \mu\text{m}$.

cells, we incubated *Naegleria* strains with mucin-coated culture plates and epithelial MDCK cells. BSM inhibited the adhesion of *N. fowleri* and *N. gruberi* trophozoites to culture plates coated with $100\ \mu\text{g ml}^{-1}$ mucin (Fig. 2a). The greatest inhibition of adhesion was observed after 30 min of incubation. *N. gruberi* adhesion was reduced from 75 to 49.8 % and adhesion of *N. fowleri* from 79.2 to 36.5 % (Fig. 2a; $P < 0.05$). Moreover, this inhibition of adhesion decreased in a time-dependent manner in both strains. Importantly, *N. fowleri* recovered the ability to adhere faster than *N. gruberi*; specifically, after 1 h of interaction, the ability to adhere was completely restored in *N. fowleri* (Fig. 2a). When PSM was used, the inhibition observed at the earlier times of incubation was stronger than that obtained with BSM; after 30 min, adhesion of *N. gruberi* was reduced from 77.4 to 21.8 % and that of *N. fowleri* from 75.8 to 30.2 % (Fig. 2b; $P < 0.05$). Similarly, as observed with BSM, the inhibition of adhesion with PSM also recovered in a time-dependent manner. Again, the capacity of *N. fowleri* to adhere returned faster than that of *N. gruberi*. As shown in Fig. 2(b), the pathogenic strain (*N. fowleri*) re-established adhesion completely after 2 h of incubation and the non-pathogen (*N. gruberi*) did so after 4 h.

We then evaluated the effect of different mucin concentrations on the inhibition of the ability of the amoebae to adhere to epithelial cells. Live trophozoites were incubated with MDCK cells in a medium supplemented with different concentrations of BSM or PSM. After 1 h of interaction, adhesion was determined as described above. Both mucins inhibited adhesion to MDCK cells in a concentration-dependent manner (Fig. 3a). The greatest inhibition was observed with $1\ \text{mg ml}^{-1}$ for both strains and both mucins tested. A $1\ \text{mg ml}^{-1}$ concentration of BSM and PSM reduced the adhesion of *N. gruberi* from 79 to 12 and

40.3 %, respectively, and from 71.6 to 35 and 37.2 %, respectively, for *N. fowleri* (Fig. 3a).

Fig. 2. Adhesion of *Naegleria* trophozoites to mucin-coated wells. (a) Comparison of *N. gruberi* and *N. fowleri* adhesion to BSM-coated (cw) or -uncoated (uw) wells after different times of incubation. (b) Comparison of *N. gruberi* and *N. fowleri* adhesion to PSM-coated (cw) or -uncoated (uw) wells after different times of incubation. Values represent the means $\pm$ SD of three replicate experiments; $*P < 0.05$, *t* test.

Fig. 3. Effect of mucins on trophozoite adhesion to MDCK cells. (a) Effect of different concentrations of BSM and PSM on *N. gruberi* (Ng) and *N. fowleri* (Nf) adhesion to MDCK cells. (b) Effect of BSM on trophozoite adhesion to MDCK cells after different periods of incubation. (c) Effect of PSM on trophozoite adhesion to MDCK cells after different periods of incubation. Values represent the means $\pm$ SD of three replicate experiments; * $P < 0.05$, t test.

As observed in the previous assays, adhesion to MDCK cells was delayed by mucins and was also restored after longer periods of incubation; nevertheless, the capacity to adhere was not completely restored. *N. gruberi* and *N. fowleri* restored their capacity to adhere to 21.2 and 53.6 %, respectively, when BSM was added, and 25.5 and 51.9 %, respectively, with PSM (Fig. 3b, c). These results suggest that mucins prevent the adhesion of the amoebae to target cells, but also that these trophozoites somehow evade this innate response by recovering the capacity to adhere in a time-dependent manner.

Mucins reduce cytotoxicity to MDCK cells

To determine the effect of mucins on amoebic cytotoxicity, live trophozoites were exposed to MDCK cells in medium supplemented with different concentrations of BSM or PSM for 12 h. After incubation, cytotoxicity was determined. Results showed that 2 mg ml⁻¹ BSM or PSM significantly reduced the *N. fowleri* cytotoxic effect by up to 58 and 61 %, respectively, after 12 h of incubation (Fig. 4b). Similar results were observed with the non-pathogenic *N. gruberi*. Both BSM and PSM reduced the *N. gruberi* cytotoxic effect to MDCK cells by up to 34 and 53 %, respectively. In the case of BSM, we observed that a lower concentration (0.5 mg ml⁻¹) reduced the damage to the cell monolayer induced by *N. gruberi* (Fig. 4a). These results are in agreement with the results of the adhesion experiments (Figs 2 and 3), in which *N. gruberi* took longer to recover the capacity to adhere to mucin-coated culture plates and to MDCK cells, and this in turn reflected the damage to the cells. Altogether, the results suggest that mucins may have an effect on amoebic adhesion and on the concomitant damage to the epithelial cells.

Mucins delay *in vivo* pathogenesis of PAM

To determine the effect of mucins on pathogenesis *in vivo*, *N. fowleri* trophozoites were inoculated by the nasal route

Fig. 4. Effect of mucins on *Naegleria* cytotoxicity to MDCK cells. Trophozoites of *N. gruberi* (a) or *N. fowleri* (b) were incubated for 12 h with MDCK cells in the presence of different concentrations of BSM or PSM. Cytotoxicity was determined by the MTT method. Values represent the means $\pm$ SD of three replicate experiments; * $P < 0.05$, t test.

into BALB/c mice in the presence of different concentrations of BSM or PSM, and then the survival curves were determined. We observed that mortality was significantly delayed in mice inoculated with live trophozoites in the presence of 5 mg ml⁻¹ BSM (Fig. 5a). In a similar manner, PSM at 5 mg ml⁻¹ significantly delayed mortality in BALB/c mice (Fig. 5b). The delay in mortality was higher using BSM as compared with PSM. Low concentrations of BSM or PSM showed no significant differences in the survival curves when compared with the control. These results suggest that mucins may participate in protection against infection with *N. fowleri*; nevertheless, the organisms must have a means of evasion, since in spite of a delay, all the mice eventually died.

Mucinolytic activity in *Naegleria* spp. trophozoites

The above results show that mucins can diminish parasitic adhesion to inert culture plates and to epithelial cells, as well as cytotoxicity. Nevertheless, the adhesion was re-established in a time-dependent manner. This phenomenon could be due to mucinolytic activity. Next, we evaluated the presence of mucinolytic activity in both pathogenic and non-pathogenic strains of *Naegleria*. Live trophozoites, total crude extracts and concentrated CM

were co-incubated with BSM or PSM for different periods of time (30 min, and 1, 3, 6, 12 and 24 h). After each incubation time, samples were electrophoresed and stained with Schiff's reagent for detection of mucins. Fig. 6(a) shows representative gels of mucins co-incubated with total extracts of *N. fowleri* and *N. gruberi*. Fig. 6(b) shows the graphic representation of three replicates of these gels analysed with optical density software. *N. fowleri* crude extracts degraded both BSM and PSM in a time-dependent manner (Fig. 6a, b). Similarly, *N. gruberi* degraded both mucins; nevertheless, when we compared the two strains, the degradation of mucins was faster with *N. fowleri* than with *N. gruberi* (Fig. 6a, b). This difference was more evident in the degradation of PSM (Fig. 6b). When 10 mM pHMB, a protease inhibitor, was added during the co-incubation, the mucinolytic activity was completely inhibited (lanes I of each gel in Fig. 6a). As a control, total crude extract without mucin was electrophoresed and stained with Schiff's reagent; no bands were detected in the control (data not shown).

Fig. 6(c) shows a graphic representation of gels of mucins co-incubated with live trophozoites. *N. fowleri* and *N. gruberi* trophozoites also showed mucinolytic activity towards both mucins tested (Fig. 6c). As with the total extracts, *N. fowleri* had a higher and faster activity than that of the non-pathogenic strain. The mucinolytic activity of total crude extracts of both strains was higher than the activity of live trophozoites (Fig. 6b, c). CM showed no mucinolytic activity, even when it was concentrated fivefold (data not shown). These results suggest that the mucinolytic activity may participate in the restoration of adhesion to epithelial cells or to culture plates observed previously, and in the evasion from mucus during mucosal epithelial tissue invasion.

Determination of mucinases in *Naegleria* spp. trophozoites

With the aim of identifying specific proteins with mucinolytic activity, total crude extracts and CM of both strains were electrophoresed in polyacrylamide gels co-polymerized with BSM or PSM and incubated at different pHs (3.0, 5.0, 7.0 and 9.0). A single band of 37 kDa with mucinolytic activity was detected in gels co-polymerized with BSM at pH 7.0 in both non-pathogenic *N. gruberi* and pathogenic *N. fowleri* (Fig. 7, lanes 1 and 2, respectively). This band was inhibited by the cysteine protease inhibitor pHMB (10 mM; Fig. 7, lanes 3 and 4). No proteolytic activity was detected in PSM co-polymerized gels. Interestingly, no activity was detected at the other pHs tested (data not shown). As in previous results, no mucinolytic bands were detected in the CM of both strains, even when these were concentrated fivefold (data not shown). These data suggest that a cysteine protease of 37 kDa may be responsible for the mucinolytic activity observed in the previous assays.

Fig. 5. Effect of mucins on PAM development in BALB/c mice. Comparison of survival curves of mice inoculated with live *N. fowleri* trophozoites in the presence of different concentrations of BSM (a) or PSM (b). Significance in survival curve differences was evaluated by the log-rank test; **P*<0.001.

Fig. 6. Determination of mucinolytic activity in total crude extracts and live trophozoites of *Naegleria* spp. Total crude extracts or live trophozoites were co-incubated with BSM or PSM and samples were withdrawn and electrophoresed at different times of incubation. (a) Representative gels of co-incubation of total crude extracts of both strains and both mucins tested. The right-hand lanes of each gel represent the co-incubation of total crude extracts and mucin for 24 h in the presence of 10 mM pHMB protease inhibitor (I). (b) Graphic representation of analysis of the above gels. (c) Graphic representation of gels obtained from the co-incubation of live trophozoites of both strains with BSM or PSM. Values represent the means $\pm$ SD of relative optical density from three separate experiments.

NCI-H292 cells and MUC5AC assays

Previously determined parameters (adhesion, cytotoxicity and mucin degradation) were tested by using a different and physiopathologically less artificial system. This was the airway epithelial cells (NCI-H292) that produce the principal known mucin of the airways, MUC5AC. The results showed that PMA (10 ng ml^{-1}) induces the expression of muc5ac mRNA, the appearance of strongly MUC5AC-positive cells and the presence of mucin in the total crude extracts and CM in comparison with the control group of non-stimulated cells (Fig. 8a, b, c). Adhesion of *N. fowleri* trophozoites to PMA-stimulated cells was substantially inhibited compared with the control (Fig. 8d). Similarly to previous results using MDCK cells, the highest inhibition was obtained at 60 min of interaction (32.6%). In the same way, amoebic cytotoxicity was significantly inhibited at 12 h of interaction (38.8%) and this inhibition was re-established in a time-dependent manner, as observed after 18 h of interaction (Fig. 8e). Finally, we tested by dot blotting the mucinolytic activity against MUC5AC of *N. fowleri* total crude extracts or live trophozoites. Results

showed that both total crude extracts and live trophozoites were able to degrade MUC5AC in a time-dependent manner (Fig. 8g). As shown for BSM and PSM, cysteine protease inhibitor was able to block the mucinolytic activity exerted by the amoebic extract (Fig. 8f). PMA-stimulated CM incubated for 24 h in the absence of amoebic extract did not show any loss of MUC5AC content (Fig. 8f). Similarly, RPMI fresh culture medium and amoebic total crude extract were negative for the presence of MUC5AC (Fig. 8f). Comparable results were obtained with the non-pathogenic strain *N. gruberi* (data not shown). These results confirmed previous experiments with BSM and PSM, and showed that the mucin MUC5AC may be an important protective barrier, reducing adhesion and cytotoxicity to the airway epithelium.

DISCUSSION

N. fowleri is a free-living amoeba that may infect human beings by inhalation from bodies of water (Marciano-Cabral & Cabral, 2007; Schuster & Visvesvara, 2004).

Fig. 7. BSM substrate gel electrophoresis incubated at pH 7.0. Thirty micrograms of total crude extracts from *N. gruberi* (lane 1) and *N. fowleri* (lane 2) were used. The effect is also shown of 10 mM pHMB cysteine protease inhibitor on the mucinolytic activity of total crude extracts from *N. gruberi* (lane 3) and *N. fowleri* (lane 4).

Histopathological studies using a murine model have shown that this amoeba infects its host by invading the olfactory mucosa, migrating through the olfactory nerves, crossing the cribriform plate, and entering the olfactory bulbs (Carter, 1970; Jarolim *et al.*, 2000, 2002). At the very early stages of infection, trophozoites are observed at the lumen of the nasal cavity embedded in mucus secretions (Rojas-Hernández *et al.*, 2004). At this stage of infection, mucin secretion may be an important protective barrier against infection. However, some trophozoites may evade this response and proceed to invade the tissues.

It is widely accepted that the mucus layer constitutes an important physical obstacle that covers the mucosal surface and protects against pathogenic micro-organisms (Moncada *et al.*, 2003b; Shirazi *et al.*, 2000). Mucins are the main glycoproteins in mucus and have special physico-chemical and protective properties (e.g. large molecular mass, high glycosylation, and polymerization) that facilitate the formation of a dense gel (Thornton & Sheehan, 2004). Therefore, parasites that invade their host via mucosal tissues must exert mechanisms that allow them to circumvent this mucous barrier. Many bacteria are known to produce proteases and glycosidases that degrade host defence components of the mucus, including mucins (Slomiany *et al.*, 1992; Mantle & Rombough, 1993). Similarly, it has been reported that some human protozoan parasites, such as *Trichomonas vaginalis*, which infects the vaginal mucosa, can pass through the mucous barrier by proteolytic degradation of mucins (Lehker & Sweeney, 1999). Moreover, the parasite *Entamoeba histolytica* can alter the protective properties of mucins by the action of cysteine proteinases (Moncada *et al.*, 2003a). This parasite

also releases abundant amounts of glycosidases that degrade colonic mucin oligosaccharides (Moncada *et al.*, 2005).

In the present study, we observed that mice are capable of secreting mucus in response to the presence of amoebae in the nasal cavity. By 6 h p.i., a strong inflammatory response is produced, mainly composed of neutrophils. Mucus and neutrophils were seen surrounding trophozoites. However, some amoebae were capable of adhering to the epithelial cells and initiating invasion of the tissues.

The effect of mucins on *Naegleria* adhesion to MDCK and MUC5AC-inducing NCI-H292 cells was examined; we found that both BSM and PSM strongly inhibited the adhesion to MDCK cells and that adhesion to MUC5AC-producing cells was also diminished. Nevertheless, the amoebae were able to overcome the inhibition of adhesion to target cells in a time-dependent manner. Interestingly, *N. fowleri* restored the capacity to adhere to target cells faster than the non-pathogenic strain. The mechanism by which mucins avoid adhesion of amoebae remains to be elucidated. In the parasite *E. histolytica*, the specific binding of colonic mucin glycoproteins to the Gal/GalNAc 170 kDa lectin present on the parasite surface is responsible for the inhibition of adhesion to colonic epithelial cells by competition with the binding components (Chadee *et al.*, 1987). We further examined the effect of mucins on amoebic cytotoxicity. As expected, mucins reduced the cytotoxic effect to target cells of pathogenic and non-pathogenic *Naegleria* trophozoites. Furthermore, inoculation of live *N. fowleri* amoebae in the presence of mucins delayed the mortality of mice. We suggest that these results are probably consequences of the effect of mucins on trophozoite adhesion.

As mentioned above, histopathological events showed that amoebae are able to overcome the initial host response and, *in vitro*, trophozoites recovered the capacity to adhere in a time-dependent manner. Therefore, we decided to evaluate the mucinolytic activity of both strains of *Naegleria* as a possible mechanism involved in the evasion of this natural immune response. Live trophozoites and total crude extracts from both species were able to degrade BSM, PSM and the airway epithelium mucin MUC5AC. The mucinolytic activity was higher in *N. fowleri* than in *N. gruberi*. These results may explain why *N. fowleri* recovers the capacity to adhere to MDCK faster than *N. gruberi*. The mucinolytic activities observed in total crude extracts were inhibited by the presence of the cysteine protease inhibitor pHMB. BSM substrate SDS-PAGE showed a 37 kDa protein with mucinolytic activity in both strains. Again, this mucinolytic band was inhibited by cysteine protease inhibitors. These results agree with a variety of earlier results in which cysteine proteases may act as mucinases (Lehker & Sweeney, 1999; Moncada *et al.*, 2003a). Although degradation of PSM was observed in both strains, bands of mucinolytic activity were not detected in gels co-polymerized with PSM. Similar results have been

Fig. 8. NCI-H292 cells and MUC5AC assays. (a) Immunocytochemistry for MUC5AC (FITC) in PMA-stimulated or non-stimulated cells; nuclei were stained with propidium iodide (red). (b) *muc5ac* mRNA expression in PMA-stimulated or non-stimulated cells; *gapdh* mRNA was used as control. (c) Dot-blot determination of MUC5AC protein expression in total crude extracts or CM of PMA-stimulated (PMA) or non-stimulated cells (Control); negative controls were lysis buffer (LB) and fresh culture medium (FM). (d) Adhesion of *N. fowleri* trophozoites to PMA-stimulated (PMA) or non-stimulated cells (Control) after different periods of incubation. (e) *N. fowleri* cytotoxicity to PMA-stimulated (PMA) or non-stimulated cells (Control) after 12 and 18 h of interaction. (f) Dot blots to MUC5AC of CM of PMA-stimulated cells (PMA), fresh culture medium (FM), CM of PMA-stimulated cells incubated alone for 24 h at 37 °C (PMA 24 h), CM of PMA-stimulated cells incubated with *N. fowleri* total extract for 24 h in the presence of 10 mM pHMB (PMA+Nf+I), and *N. fowleri* total extract alone (Nf). (g) Dot blots to MUC5AC of PMA-stimulated cells CM co-incubated for different time periods with *N. fowleri* total crude extract (PMA+Nf extract) or live trophozoites (PMA+Live Nf).

obtained with the protozoan parasite *T. vaginalis* (Lehker & Sweeney, 1999); those authors reported that *T. vaginalis* is able to degrade PSM, although no mucinolytic band in gels co-polymerized with this mucin could be detected. Mucinolytic activity was not detected in CM of both *Naegleria* strains, despite the fact that secreted cysteine proteases have been reported to be present for *N. fowleri* and *N. gruberi* (Aldape *et al.*, 1994; Serrano-Luna *et al.*, 2007); it is likely that these proteases do not have specificity for the degradation of mucins.

This study is believed to represent the first report of the presence of mucinolytic activity in free-living amoebae and suggests that *N. fowleri* cysteine proteases may act as a part of the pathogenic mechanism by degrading the mucins present on the mucous surface of the nasal epithelium. A

study with *E. histolytica* trophozoites has shown that the use of an antisense transcript of cysteine proteases with mucinolytic activity abrogates the capacity to overcome a protective intact mucus barrier (Moncada *et al.*, 2006). Molecular characterization of this mucinolytic activity is now required for elucidation of its importance in the pathogenesis of PAM produced by *N. fowleri*.

In most cases, the complex structure of mucous secretion, which includes mucins and other components, such as sIgA, defensins, lysozyme and lactoferrin, is an effective barrier that traps and eliminates pathogenic micro-organisms (Moncada *et al.*, 2003b). However, when microbial colonization and mucus degradation rates exceed the mucus response, some parasites are able to invade the tissues (Moncada *et al.*, 2003b). Finally, we suggest that mucous

secretion also delays amoebic adhesion (Shibayama *et al.*, 2003), and may represent a protective immune response that prevents most infections by this free-living amoeba. This feature agrees with the observation that despite the wide ecological distribution of this amphizoic amoeba, the incidence of infection is relatively low. Nevertheless, when the number of infective amoebae is large, the parasites can overwhelm the mucus response by degrading the mucins by means of mucinolytic cysteine proteases. This is further substantiated by the fact that most cases of PAM occur in the hottest months of the year (Yoder *et al.*, 2004), when amoebic populations increase (Kyle & Noblet, 1986).

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