Naegleria fowleri induces MUC5AC and pro-inflammatory cytokines in human epithelial cells via ROS production and EGFR activation

Isaac Cervantes-Sandoval,^{1,3} José de Jesús Serrano-Luna,² Patricia Meza-Cervantez,¹ Rossana Arroyo,¹ Víctor Tsutsumi¹ and Mineko Shibayama¹

Correspondence

Mineko Shibayama
mineko@cinvestav.mx

¹Department of Infectomic and Molecular Pathogenesis, Center for Research and Advanced Studies, National Polytechnic Institute, Av. IPN 2508, Mexico City 07360, Mexico

²Department of Cell Biology, Center for Research and Advanced Studies, National Polytechnic Institute, Av. IPN 2508, Mexico City 07360, Mexico

³Department of Immunology, National School of Biological Sciences, National Polytechnic Institute, Av. Manuel M. Carpio and Plan de Ayala, Mexico City 11340, Mexico

Naegleria fowleri is an amoeboflagellate responsible for the fatal central nervous system (CNS) disease primary amoebic meningoencephalitis (PAM). This amoeba gains access to the CNS by invading the olfactory mucosa and crossing the cribriform plate. Studies using a mouse model of infection have shown that the host secretes mucus during the very early stages of infection, and this event is followed by an infiltration of neutrophils into the nasal cavity. In this study, we investigated the role of *N. fowleri* trophozoites in inducing the expression and secretion of airway mucin and pro-inflammatory mediators. Using the human mucoepidermal cell line NCI-H292, we demonstrated that *N. fowleri* induced the expression of the MUC5AC gene and protein and the pro-inflammatory mediators interleukin-8 (IL-8) and interleukin-1 β (IL-1 β), but not tumour necrosis factor- α or chemokine c-c motif ligand 11 (eotaxin). Since the production of reactive oxygen species (ROS) is a common phenomenon involved in the signalling pathways of these molecules, we analysed if trophozoites were capable of causing ROS production in NCI-H292 cells by detecting oxidation of the fluorescent probe 2,7-dichlorofluorescein diacetate. NCI-H292 cells generated ROS after 15–30 min of trophozoite stimulation. Furthermore, the expression of MUC5AC, IL-8 and IL-1 β was inhibited in the presence of the ROS scavenger DMSO. In addition, the use of an epidermal growth factor receptor inhibitor decreased the expression of MUC5AC and IL-8, but not IL-1 β . We conclude that *N. fowleri* induces the expression of some host innate defence mechanisms, such as mucin secretion (MUC5AC) and local inflammation (IL-8 and IL-1 β) in respiratory epithelial cells via ROS production and suggest that these innate immune mechanisms probably prevent most PAM infections.

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INTRODUCTION

Naegleria fowleri is an ubiquitous, free-living amoeba that has been isolated from diverse water and soil sources. Under certain conditions, which are not fully understood, these amoebae may infect the human central nervous

system (CNS), giving rise to primary amoebic meningoencephalitis (PAM). Infection usually occurs in young healthy people with a history of recent recreational water activities (Marciano-Cabral & Cabral, 2007). Despite the ubiquity and worldwide distribution of these amoebae, only a little over 200 cases have been reported worldwide. Trophozoites invade the host by penetrating the olfactory neuroepithelium and migrating through the cribriform plate, before reaching the olfactory bulbs (Carter, 1970; John & Nussbaum, 1983; Jarolim *et al.*, 2000; Rojas-Hernández *et al.*, 2004). Once in the CNS, the amoebae induce a strong inflammatory reaction, haemorrhage and lytic necrosis (Carter, 1970; Cervantes-Sandoval *et al.*, 2008a).

Abbreviations: CCL11, chemokine c-c motif ligand 11; CNS, central nervous system; EGFR, epidermal growth factor receptor; FDA, 2,7-dichlorofluorescein diacetate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-1 β , interleukin-1 β ; IL-8, interleukin-8; MUC5AC, mucin 5AC; PAM, primary amoebic meningoencephalitis; PMA, phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; TGF- α , transforming growth factor- α ; TNF- α , tumour necrosis factor- α .

Immunohistochemical studies during the initial stages of infection have shown the presence of several trophozoites embedded within the mucus in the nasal cavity. This event is followed by the appearance of inflammatory cells, mainly neutrophils, surrounding the amoebae (Rojas-Hernández *et al.*, 2004; Cervantes-Sandoval *et al.*, 2008b). In order to improve our understanding of the initial stages of infection, it is necessary to study the molecular mechanisms involved in the activation of the inflammatory cells and other innate immune responses, and how these responses may either protect from or contribute to the pathogenesis of PAM.

In the present study, we evaluated the ability of *N. fowleri* trophozoites to induce the expression of the MUC5AC gene and protein, and the pro-inflammatory cytokines interleukin-8 (IL-8), interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF- α) and the unrelated eosinophil chemokine c-c motif ligand 11 (CCL11; eotaxin). In addition, we investigated some of the signalling routes involved in this activation, such as the generation of reactive oxygen species (ROS) and activation of epidermal growth factor receptor (EGFR). We used the intensively studied human airway epithelial cell line NCI-H292, which expresses the main human and mouse airway mucin MUC5AC and pro-inflammatory cytokines, in response to certain stimuli (Shao *et al.*, 2003; Shao & Nadel, 2005; Nakanaga *et al.*, 2007). The results showed that *N. fowleri* trophozoites induced the expression of MUC5AC, IL-8 and IL-1 β , but not TNF- α or CCL11, in NCI-H292 cells. The expression was time-dependent, with peaks at 6 and 12 h following stimulation. *N. fowleri* trophozoites also induced the production of ROS after 30 and 60 min. MUC5AC, IL-8 and IL-1 β expression were inhibited by the ROS scavenger DMSO, confirming the involvement of these mediators in the signalling cascade leading to the expression of these innate immune response products. Finally, the expression of MUC5AC and IL-8, but not IL-1 β , was blocked by a specific inhibitor of EGFR, as previously reported, using phorbol 12-myristate 13-acetate (PMA) as a stimulus (Nakanaga *et al.*, 2007). These results suggest that *N. fowleri* trophozoites induce innate immune responses in epithelial cells, such as NCI-H292, during the early stages of infection. Secretion of mucins and expression of pro-inflammatory cytokines are some of these responses, which are at least partially dependent on ROS generation and EGFR activation.

METHODS

Amoebic and cell cultures. The pathogenic strain *N. fowleri* ATCC 30808 was used in all experiments. Trophozoites were axenically cultured in 2% (w/v) bactocitine medium supplemented with 10% (v/v) fetal bovine serum (FBS; Equitech-bio) at 37 °C. Trophozoites were harvested during the exponential growth phase (48 h).

NCI-H292 cells, which have been widely used as a model for studies of mucin and innate response generation, were grown in RPMI 1640 medium with 10% (v/v) FBS (Gibco) and 25 mM HEPES at 37 °C, in

a 5% CO₂ atmosphere. After reaching semi-confluence (80%; 5×10^5 cells per six-well culture plate), 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 different stimuli.

Experimental animals. Experiments with animals were performed as previously described (Cervantes-Sandoval *et al.*, 2008a). Briefly, 4-week-old male BALB/c mice were lightly anaesthetized in a chamber, using ether, and inoculated by the nasal route with 2.5×10^4 live *N. fowleri* trophozoites in 20 μ l. After different post-inoculation intervals (1, 6 and 12 h), the animals were euthanized and the brains were fixed. The skulls were decalcified and the brain tissue was processed and embedded in paraffin wax. Sections were cut at 7 μ m and stained using a rabbit polyclonal anti-*N. fowleri* antibody produced in our laboratory [diluted 1:50 in PBS-Tween 0.05% (v/v)] and a secondary antibody, anti-rabbit IgG labelled with peroxidase (diluted 1:200; Sigma-Aldrich). Colour was developed using a diaminobenzidine kit (Pierce).

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.

Expression of MUC5AC and pro-inflammatory cytokines. To determine if *N. fowleri* trophozoites were able to induce an innate immune response in the mucoepithelial NCI-H292 cells, serum-starved cells were co-incubated with 5×10^5 amoebae at a ratio of 1:1, for 1–6 h. After 6 h, amoebae were removed by cooling the medium for 5 min, and the incubation continued up to 12 and 24 h. This procedure was necessary because co-incubation with amoebae for longer than 6 h resulted in damage of the cell monolayer. *N. fowleri*-conditioned medium was prepared as described previously (Serrano-Luna *et al.*, 2007) and was added to NCI-H292 cells for different time periods (1, 3, 6, 12 and 24 h). For positive controls, cells were stimulated with PMA, 10 ng ml⁻¹ for 1 h, then washed three times with serum-free medium and cultured for an additional 24 h. For negative controls, cells were incubated in RPMI medium without serum for 24 h (to maintain basal levels of expression). Finally, cell culture supernatants and cell lysates were collected to measure the expression of MUC5AC protein and cytokine mRNAs (IL-8, IL-1 β , TNF- α and CCL11).

MUC5AC protein determination. MUC5AC protein was determined by immunocytochemistry and dot-blots. For immunocytochemistry, stimulated and non-stimulated cells were fixed with 2% (w/v) paraformaldehyde and stained with mouse monoclonal antibody to MUC5AC (clone 1-13M1, Abcam) and our rabbit polyclonal anti-*N. fowleri* antibody (see above). The secondary antibodies used were goat anti-mouse IgG labelled with FITC (Zymed) and goat anti-rabbit IgG labelled with Cy5 (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-blots, 2 μ l samples of total crude cell lysates (protein concentration adjusted to 5 μ l ml⁻¹) or conditioned cultured medium were absorbed onto a nitrocellulose membrane and blocked with 5% (w/v) skimmed milk for 1 h. Then the membrane was washed four times with PBS-Tween at 0.05% (v/v) and incubated with the same monoclonal antibody to MUC5AC for 2 h. The membrane was washed four times and incubated with a secondary antibody against mouse IgG labelled with horseradish peroxidase. Finally the membrane was washed six times and revealed with luminol kit reagent (Santa Cruz Biotechnology). As controls, fresh RPMI culture medium or lysis buffer were tested for

the presence of MUC5AC; these tests gave negative results. The optical density of the dots was analysed using the NIH image software ImageJ (<http://rsb.info.nih.gov/ni-image>). Results are presented as one representative blot with the densitometry analysis of at least three replicates with similar results.

Analysis of MUC5AC and cytokine mRNA expression by RT-PCR. In order to evaluate the levels of mucin and cytokine messenger expression after stimulation, total RNA was isolated using the QIAamp RNA blood mini kit (Qiagen), according to the manufacturer's procedure. mRNA expression was then analysed by RT-PCR using the One-Step RT-PCR kit (Qiagen), according to the manufacturer's instructions, and using the specific primers for MUC5AC, IL-8, IL-1 β , TNF- α , CCL11 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) listed in Table 1. Primers without references were designed using the software Primer3 plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). PCR products were analysed by electrophoresis in 2 % (w/v) agarose gels, stained with ethidium bromide and visualized in a UV transilluminator (Bio-Rad). To measure the expression levels, bands in gels were analysed using the ImageJ. Results are presented as one representative gel with the densitometry analysis of at least three replicates with similar results. All PCR products were purified and sequenced to verify their identity. Briefly, the PCR products were polyadenylated, inserted in the pCR4-TOPO plasmid (Invitrogen), and used to transform *Escherichia coli* DH5 α . Bacteria were seeded in solid LB medium containing ampicillin (5 μ g ml⁻¹) and grown for 24 h. Further selection of positive colonies was made by PCR. Positive bacteria were again grown in liquid LB medium and the plasmid was purified and sequenced using the M13 sequencing primers. The results showed that the sequences were 100 % homologous to the predicted sequences (data not shown).

ROS analysis. We determined the capacity of *N. fowleri* trophozoites to generate ROS in NCI-H292 cells. NCI-H292 cells were grown in six-well culture plates until confluence was reached. They were then maintained in RPMI culture medium without serum for 24 h prior to stimulation. The cells were stimulated with 5 $\times$ 10⁵ trophozoites (1 : 1 ratio, as mentioned above), or 10 ng PMA ml⁻¹ as a positive control, for 15, 30 or 60 min in RPMI without serum and supplemented with 30 μ M 2,7-dichlorofluorescein diacetate (FDA). After the interaction time, the cell monolayers were washed four times with cold PBS to remove trophozoites and disaggregated using trypsin for 5 min. Finally, they were washed three times with PBS and fluorescence was quantified in a fluorometer with an excitation wavelength of 485 nm and emission at 525 nm. The fluorescence emitted by the non-stimulated cells was taken as the baseline level of ROS. The results are reported as mean $\pm$ SD of the increase over baseline value. The significance of the differences was evaluated using *t*-test, *P* < 0.05.

Effect of DMSO and tyrphostin AG1478. We examined the roles of ROS and activation of the EGFR in the expression of pro-inflammatory cytokines and mucin. Cells were incubated with trophozoites for 6 h, then they were removed from the culture medium and the incubation continued up to 24 h, as mentioned above, in the presence of 1 % (v/v) DMSO, a compound that captures and inhibits the activity of ROS. In parallel assays, the cells were pre-incubated with 10 μ M tyrphostin AG1478, a strong inhibitor of EGFR, for 30 min before the stimulus with amoebae, as mentioned in the previous assay. After inhibition, cells were interacted with the stimuli and MUC5AC and cytokine expression were analysed as described above.

RESULTS

N. fowleri induces mucus secretion and neutrophil infiltration in the mouse nasal cavity

To corroborate previous observations of experimental PAM during the early stages of infection, we inoculated the nasal cavities of mice with live *N. fowleri* trophozoites and determined the induction of mucus secretion. The host mucosa reacted as early as 1 h post-inoculation by strong production and secretion of mucus, as previously reported (Rojas-Hernández *et al.*, 2004; Cervantes-Sandoval *et al.*, 2008b) (data not shown). Before contacting the epithelial cells, some trophozoites were intermixed with mucus in the lumen of the nasal cavity. Six hours post-inoculation, amoebae were seen covered by the mucus secretion and surrounded by a strong acute inflammatory reaction, composed mainly of neutrophils. Control animals inoculated with fresh culture medium showed no changes in the olfactory neuroepithelium (data not shown). These results suggest that the mouse epithelial cells responded to amoebic infection by exerting an innate immune response characterized by the production of mucus and pro-inflammatory signals. We used an *in vitro* approach to further elucidate the mechanisms involved in this response.

N. fowleri induces the expression of MUC5AC in human mucoepithelial cells

We evaluated the ability of *N. fowleri* trophozoites to induce the expression of mucins, particularly MUC5AC,

Table 1. RT-PCR primers

Protein encoded	Primer A	Primer B	Annealing temp. (°C)	Reference
MUC5AC	5'-CAC CAA ATA CGC CAA CAA GAC-3'	5'-CAG GGC CAC GCA GCC AGA GAA-3'	61	Shimizu <i>et al.</i> (2003)
IL-8	5'-ATG ACT TCC AAG CTG GCC GTG-3'	5'-CTC AGC CCT CTT CAA AAA CTT-3'	58	Teruya <i>et al.</i> (2007)
TNF- α	5'-TCC TTC AGA CAC CCT CAA CC-3'	5'-AGG CCC CAG TTT GAA TTC TT-3'	53	Primer3 plus
IL-1 β	5'-GGG CCT CAA GCA AAA GAA TC-3'	5'-TTC TGC TTG AGA GGT GCT GA-3'	55	Primer3 plus
CCL11	5'-AGA AAC CAC CAC CTC TCA CG-3'	5'-CAC AGC TTT CTG GGG ACA TT-3'	55	Primer3 plus
GAPDH	5'-CCA CCC ATG GCA AAT TCC ATG GCA-3'	5'-TCT AGA CGG CAG GTC AGG TCC ACC-3'	66	Shimizu <i>et al.</i> (2003)

which is the most abundant mucin in the airway epithelium in humans and mice, and has been reported to be overexpressed following diverse chemical and biological stimuli (Shao *et al.*, 2003). We used NCI-H292 cells, which have been widely used to study upper-airway mucins, including MUC5AC, and other innate immune mediators. NCI-H292 cells were co-incubated with conditioned medium or *N. fowleri* trophozoites for different periods of time; the production of MUC5AC protein was determined by immunocytochemistry and dot-blots, and

the expression of mRNA by RT-PCR. Confocal microscopy using a secondary antibody coupled to FITC showed that *N. fowleri* was able to strongly induce MUC5AC, compared with control, non-stimulated cells, which showed no MUC5AC-positive cells (Fig. 1a). MUC5AC production was apparent after 3 h interaction with trophozoites stained with a secondary antibody coupled to Cy5 (blue), and expression increased up to 24 h of co-incubation (Fig. 1a). The determination of MUC5AC protein by dot-blots indicated that *N. fowleri* was able to induce MUC5AC

Fig. 1. MUC5AC expression in NCI-H292 epithelial cells induced by *N. fowleri*. (a) Confocal microscopy shows the production of MUC5AC (FITC) by NCI-H292 cells stimulated by trophozoites (Cy5, *Nf*) or PMA, and by non-stimulated cells (control), after 3, 6, 12 and 24 h. Cells were incubated with trophozoites for 6 h, then they were removed from the culture medium and the incubation was continued up to 12 and 24 h. Nuclei were stained with propidium iodide. Scale bars, 20 µm. (b) MUC5AC protein expression determined by dot-blots after stimulation with *N. fowleri* trophozoites. Negative controls were serum-complemented fresh medium (FM) and *N. fowleri* crude extract (*Nf*); control cells without stimulus were incubated in medium without serum (SS) and cells stimulated with PMA were used as positive expression control. Densitometric analysis results are shown below the blots. (c) Expression of MUC5AC RNA determined by RT-PCR after stimulation with *N. fowleri* trophozoites for different time points. Control cells without stimulus were incubated in medium without serum (SS) and cells stimulated with PMA were used as positive expression control. Densitometric analysis results are shown below the blots.

expression in a time-dependent manner, reaching a maximum expression level of almost sixfold that of the control by 12 h after stimulation, which was maintained at 24 h (Fig. 1b). In the positive (PMA) control, the dot-blot showed an overproduction of almost 12-fold, in accordance with the confocal analysis. The non-stimulated, negative control (SS) showed very low basal production of MUC5AC protein (Fig. 1b). Similarly, we tested the fresh medium and the total amoebic crude extract for the presence of MUC5AC protein, giving negative results (Fig. 1b). Analysis of mRNA expression showed similar results. *N. fowleri* trophozoites induced the expression of MUC5AC mRNA in a time-dependent manner. Expression became apparent from 3 h and increased to reach a maximum of approximately 12-fold after 6 and 12 h (Fig. 1c). In contrast to protein analysis, the positive control showed a marked increase in expression (ninefold), but it was lower than the level induced by *N. fowleri*. This could be related to the different mechanisms of control and regulation of expression, for example the stability of mRNA. Nevertheless, further analysis is required to clarify these observations. As an amplification control, the reactions were adjusted to GAPDH mRNA expression (Fig. 1c). These results suggest that mucin secretion in NCI-H292 cells is an active process induced by the presence of *N. fowleri*.

***N. fowleri* induces the expression of pro-inflammatory signals in human mucoepithelial cells**

Because the animal model shows an inflammatory infiltrate with secretion of mucus in the nasal cavity in response to

N. fowleri, we evaluated the effect of trophozoites on the induction of mRNA for some pro-inflammatory cytokines (IL-8, TNF- α , IL-1 β , CCL11) in NCI-H292 cells. *N. fowleri* trophozoites were co-incubated with NCI-H292 cells for different time periods and the mRNA expression of the different cytokines was determined. The amoebae induced the expression of IL-8 from 3 h (threefold), as for MUC5AC, and expression continued to increase to a maximum level of 16-fold after 24 h of interaction (Fig. 2a, b), which was very similar to the expression pattern observed in the PMA-treated, positive control cells. Similarly, *N. fowleri* induced the expression of IL-1 β after 3 h of interaction (fivefold), and levels increased to almost 13-fold after 24 h. The highest level of IL-1 β obtained after *N. fowleri* stimulation was lower than the levels obtained with PMA, which resulted in an increase of about 16-fold over the control. We observed basal expression levels of the pro-inflammatory cytokine TNF- α under all conditions studied, and this expression was not modified by stimulation with *N. fowleri* trophozoites (Fig. 2a, b). In contrast, PMA was able to induce a strong expression of TNF- α . Finally, we evaluated expression of the unrelated pro-inflammatory cytokine CCL11, which is a strong chemotactic stimulator of eosinophils, which are not observed *in vivo* during the early stages of infection. As expected, neither *N. fowleri* trophozoites nor PMA were able to increase the production of CCL11 (Fig. 2a); we did not test alternative stimuli to demonstrate expression of CCL11 in these cells. As a positive control, we used genomic DNA to amplify CCL11 under the same RT-PCR conditions, and obtained a product of the predicted size (Fig. 2a). These results indicate that *N. fowleri* induced the expression of the pro-inflammatory cytokines IL-1 β and

Fig. 2. *N. fowleri* induces the expression of pro-inflammatory cytokines in NCI-H292 epithelial cells. (a) Determination of expression levels of IL-8, IL-1 β , TNF- α , CCL11 and GAPDH (housekeeping) mRNAs by RT-PCR, after different periods of stimulation with *N. fowleri* trophozoites. Cells were incubated with trophozoites for 6 h, then they were removed from the culture medium and the incubation was continued up to 24 h. Negative control cells were cultured in medium without serum (SS) and positive control cells were incubated with 10 ng PMA ml⁻¹. PCR of genomic DNA was used to validate the RT-PCR conditions. (b) Densitometric analysis of the bands obtained in (a). Results represent the expression levels of the different cytokines relative to the control.

IL-8 in epithelial cells, and correlate with the infiltration of neutrophils into the nasal cavity observed in the animal model, since these are strong neutrophil-chemotactic molecules. To validate the amplified products, PCR was performed using genomic DNA to ensure that the conditions of the amplification reaction were appropriate; these controls were always positive (Fig. 2a). Similarly, all amplified fragments were purified and sequenced to verify their identities. The sequencing of amplicons showed 100 % homology with sequences reported for the different mRNAs.

N. fowleri increases production of ROS

Because ROS have been reported to play a key role in regulating the expression of MUC5AC, IL-1 β and IL-8, we determined if *N. fowleri* induced an increase in ROS and if this increase was involved in regulating the synthesis of these molecules. *N. fowleri* trophozoites were co-incubated with NCI-H292 cells for 15, 30 or 60 min in the presence of the ROS fluorescent probe FDA. ROS were slightly increased 15 min after addition of *N. fowleri*, but marked increases of up to 1.6 times that of the non-stimulated control cells occurred after 30 min of interaction, and up to 2.3 times after 60 min (Fig. 3). Positive control cells stimulated with PMA showed increases of up to 1.6, 1.9 and 3.4 times after 15, 30 and 60 min of interaction, respectively (Fig. 3). These results strongly suggest that an increase in ROS may be involved in the positive regulation of cytokines and mucin MUC5AC.

ROS production and EGFR activation are required for MUC5AC and cytokine overexpression

As mentioned previously, ROS generation has been reported to be important for the upregulation of several

Fig. 3. *N. fowleri* induces the production of ROS. ROS were determined by FDA fluorescence in NCI-H292 cells stimulated with PMA as positive control, or with *N. fowleri* trophozoites, for different time periods (15, 30 and 60 min). Data show the production of ROS relative to that in control, non-stimulated cells. Values represent the means $\pm$ SD of three replicate experiments; * $P < 0.05$ %, t -test.

molecules involved in innate immune responses (Hsu & Wen, 2002). The above results suggest that the increase in ROS observed a few minutes after *N. fowleri* stimulation may be involved in the upregulation of MUC5AC, IL-8 and IL-1 β . To test this hypothesis, we stimulated NCI-H292 cells with trophozoites for 6 h, then the amoebae were removed and the incubation was continued for 24 h in the presence of 1 % DMSO (a ROS scavenger). After the interaction, we measured the mRNA expression of the molecules previously tested. Inhibition of the activity of ROS with DMSO reduced the expression levels of MUC5AC, IL-8, IL-1 β to 0 %, 21 % and 32 %, respectively, when expression levels in the absence of DMSO were taken as 100 % (Fig. 4a, b). Similar results were obtained in cells interacted with PMA as a positive control (data not shown). These results indicate that *N. fowleri* is able to increase the levels of ROS, and that this increase is significant and is probably involved in the signalling pathway leading to increased expression of MUC5AC, IL-8 and IL-1 β .

It has also been shown that ROS are involved in the signalling pathway leading to IL-8 and MUC5AC production, by activation of EGFR. To demonstrate if activation of this receptor is involved in the expression of MUC5AC, IL-1 β and IL-8 induced by *N. fowleri*, NCI-H292 cells were stimulated with trophozoites as in the previous assay, after pre-incubation for 30 min with 10 μ M tyrphostin AG1478, a specific inhibitor of EGFR phosphorylation (activation). Total RNA was extracted from the cells and the levels of MUC5AC, IL-8 and IL-1 β expression were analysed by RT-PCR. MUC5AC and IL-8 expression levels were reduced after specific inhibition of EGFR. MUC5AC levels were reduced to 0 %, and IL-8 levels to 41 % (Fig. 4a, b). Again, similar results were obtained in cells interacted with PMA as a positive control (data not shown). These results indicate that EGFR activation is necessary to induce the production of these proteins. In contrast, the expression of IL-1 β was not inhibited by tyrphostin AG1478, indicating that EGFR is not involved in regulating the expression of this cytokine (Fig. 4a, b). When we combined both inhibitors (DMSO and tyrphostin), the results were similar to those obtained with only DMSO: low expression of IL-8 and IL-1 β was still observed (Fig. 4a, b). This result indicates that the effect of these inhibitors is not synergistic, and perhaps another kind of receptor or signalling pathway participates in the production of these pro-inflammatory cytokines.

DISCUSSION

N. fowleri is a free-living amoeboflagellate that can infect humans by invading the nasal tissues. The infection usually occurs after performing aquatic activities in water bodies containing the parasite (Schuster & Visvesvara, 2004; Marciano-Cabral & Cabral, 2007). Histopathological studies using a mouse model showed that the amoebae infect the host by penetrating the olfactory mucosa,

Fig. 4. Role of ROS and EGFR in MUC5AC and cytokine expression. (a) RT-PCR determination of the effect of DMSO (ROS scavenger), tyrophostin (EGFR inhibitor) or both (D + T) on the expression of MUC5AC and pro-inflammatory cytokines in NCI-H292 cells after interaction with *N. fowleri* trophozoites. (b) Densitometric analysis of the gels in (a), showing the percentage expression of the different mRNAs, relative to the expression levels observed in cells stimulated without the presence of any inhibitor (100 %).

migrating through the olfactory nerves, and passing through the cribriform plate to reach the olfactory bulbs of the CNS, where an intense inflammatory reaction is produced (Carter, 1970; Jarolim *et al.*, 2000, 2002; Cervantes-Sandoval *et al.*, 2008a). In the very early stages of infection, trophozoites are seen in the lumen of the nasal cavity embedded in a large amount of mucus and surrounded by infiltrated neutrophils (Thong *et al.*, 1983; Rojas-Hernández *et al.*, 2004; Cervantes-Sandoval *et al.*, 2008b). At this stage of infection, mucin secretion, inflammation and other local innate responses could be important protective barriers against infection. It has been reported that the presence of humoral (Edwards *et al.*, 1976) and cellular immunity (Cursons *et al.*, 1980) may be protective against *Naegleria* infections. This protection is probably mediated by primed neutrophils killing trophozoites, in which antibodies play an important role in the opsonization of the parasites (Ferrante & Mocatta, 1984; Ferrante, 1989); the alternative activation pathway of the complement could also be involved (Rowan-Kelly *et al.*, 1980).

In this study, we determined if the processes of mucin hypersecretion and inflammation observed in the animal model were active processes stimulated directly by the presence of the parasite. We used a widely studied *in vitro* model comprising mucin production by NCI-H292 cells, an immortal cell line derived from a human cancer, which responds to a variety of chemical and biological stimuli such as H₂O₂ (Takeyama *et al.*, 2000) and LPS (Smirnova *et al.*, 2003). Although olfactory epithelium does not usually contain mucin-producing goblet cells, this epithelium is a relatively small and limited region at the roof of the nasal cavity and behind the nostrils, and is closely associated with an epithelium of the upper respiratory type that contains abundant goblet cells. So mucin secretion by

these cells could be important, since trophozoites must pass the respiratory epithelium in the nasal cavity before approaching and invading the olfactory epithelium.

We showed that *N. fowleri* was capable of inducing the expression and secretion of MUC5AC *in vitro*. In a previous report, we demonstrated that MUC5AC and other mucins could be important resistance factors protecting against *N. fowleri* infection by inhibiting adhesion (Cervantes-Sandoval *et al.*, 2008b). MUC5AC is the most abundant mucin in the upper respiratory tract and is secreted mainly by goblet cells or ciliated epithelial cells differentiated to mucin-producing cells (Rose *et al.*, 2001; Kim *et al.*, 2005). It polymerizes to form a thick gel with protective properties. Recent studies have suggested that ROS play a key role in the signalling pathways involved in inducing the expression of MUC5AC (Takeyama *et al.*, 2000). Stimulation of NCI-H292 cells with H₂O₂ activates the production of ROS, leading to the expression of MUC5AC (Takeyama *et al.*, 2000) via activation of the EGFR (Takeyama *et al.*, 1999). In order to determine if this signalling pathway is involved in the expression of MUC5AC induced by *N. fowleri*, we measured ROS in cells stimulated with this amoeba. The results showed that *N. fowleri* increased the production of ROS in the epithelial cell line NCI-H292, starting after a few minutes of interaction. This result suggests that ROS could be involved in the activation of the cellular response by the external agent (amoebae), and could be important in activating various signalling pathways, including those leading to the production of MUC5AC. To prove this hypothesis, we determined the importance of ROS in the induction of MUC5AC, by treating NCI-H292 cells with the ROS scavenger DMSO. During stimulation with amoebae, DMSO significantly decreased the expression of MUC5AC induced by *N. fowleri* or PMA, so confirming the

involvement of ROS in regulating mucin expression. The most important outcome of the increase in ROS during the regulation of mucin expression is the activation of EGFR (Takeyama *et al.*, 2000). Pre-incubation of cells with DMSO for 30 min prior to stimulation with amoebae abolished the production of MUC5AC. These results confirmed that stimulation with *N. fowleri* leads to an increase in ROS, which in turn leads to activation of the EGFR, which is involved in the regulation of MUC5AC. Similarly, *N. fowleri* was found to induce the expression of the pro-inflammatory cytokines IL-1 β and IL-8. This induction is in accordance with the strong inflammatory infiltrate, composed mainly of neutrophils, present in the nasal cavity prior to the invasion of tissues. IL-8 is one of the strongest neutrophil chemoattractants (Ibelgafts, 1995) and specifically activates these inflammatory cells. Here, we showed that both tyrphostin and DMSO significantly inhibited the expression of IL-8 in NCI-H292 cells, indicating that *N. fowleri* stimulates the production of IL-8 and MUC5AC by a shared mechanism as reported by Nakanaga *et al.* (2007).

On the other hand, IL-1 β is a molecule with various functions and is an important mediator of inflammatory reactions. The *in vivo* injection of this cytokine induces the local accumulation of neutrophils at the site of inoculation, as well as the activation of oxidative metabolism of neutrophils (Ibelgafts, 1995). Similar to the situation for MUC5AC and IL-8, ROS have been shown to be involved in the regulation of IL-1 β (Hsu & Wen, 2002). Moreover, it has been determined that IL-1 is positively regulated through different signalling pathways, which may depend on p38, JNK or MEK, according to the stimulus, but that all the pathways are dependent on an increase in ROS (Kim *et al.*, 2004). In the present study, we showed that inhibition of ROS by DMSO also reduced the expression of IL-1 β after stimulation by the amoebae. However, unlike the situation with MUC5AC and IL-8, EGFR inhibition did not affect the expression of IL-1 β , suggesting that these molecules do not share the same signalling pathway, except for their dependence on ROS generation. Furthermore, it was noted that *N. fowleri* was incapable of inducing the expression of CCL11, as expected, since CCL11 is an eosinophil chemoattractant, and no eosinophils are observed in the nasal cavity *in vivo*. In contrast to positive control cells stimulated with PMA, *N. fowleri* trophozoites were incapable of inducing TNF- α . Production of this molecule is increased in acute events triggered by different stimuli, and it has been shown to be involved in the production of mucin in the nasal epithelium. PMA must induce signalling pathways that are different from those produced by *N. fowleri*. Nevertheless, these results need to be confirmed using different epithelial cell lines or primary culture cells, since immortal cell cultures are not always representative of the *in vivo* function.

In conclusion, we have determined that *N. fowleri* is capable of activating epithelial cells and that this activation leads to increased ROS production, which in turn helps to

increase the expression of MUC5AC and IL-8 by an EGFR-dependent mechanism and of IL-1 β by an EGFR-independent mechanism. These results are consistent with observations at early stages of infection in the *in vivo* model, characterized by the presence of parasites in the nasal cavity surrounded by abundant mucus and inflammatory infiltrates rich in neutrophils. We propose that these initial natural immune responses, together with specific cellular and humoral immunity (Cursons *et al.*, 1980), including activated neutrophils (Ferrante & Mocatta, 1984; Ferrante, 1989; Ferrante *et al.*, 1988, 1989) and the alternative activation of complement (Rowan-Kelly *et al.*, 1980) may all participate in the prevention of most *N. fowleri* infections. More studies are needed to further increase our understanding of the early events occurring during PAM pathogenesis and the means by which the host generates an effective innate response against *N. fowleri* invasion.

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