Development of a high- versus low-pathogenicity model of the free-living amoeba Naegleria fowleri

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Species in the genus Naegleria are free-living amoebae of the soil and warm fresh water. Although around 30 species have been recognized, Naegleria fowleri is the only one that causes primary amoebic meningoencephalitis (PAM) in humans. PAM is an acute and fast progressing disease affecting the central nervous system. Most of the patients die within 1–2 weeks of exposure to the infectious water source. The fact that N. fowleri causes such fast progressing and highly lethal infections has opened many questions regarding the relevant pathogenicity factors of the amoeba. In order to investigate the pathogenesis of N. fowleri under defined experimental conditions, we developed a novel high- versus low-pathogenicity model for this pathogen. We showed that the composition of the axenic growth media influenced growth behaviour and morphology, as well as in vitro cytotoxicity and in vivo pathogenicity of N. fowleri. Trophozoites maintained in Nelson’s medium were highly pathogenic for mice, demonstrated rapid in vitro proliferation, characteristic expression of surface membrane vesicles and a small cell diameter, and killed target mouse fibroblasts by both contact-dependent and -independent destruction. In contrast, N. fowleri cultured in PYNFH medium exhibited a low pathogenicity, slower growth, increased cell size and contact-dependent target cell destruction. However, cultivation of the amoeba in PYNFH medium supplemented with liver hydrolysate (LH) resulted in trophozoites that were highly pathogenic in mice, and demonstrated an intermediate proliferation rate in vitro, diminished cell diameter and contact-dependent target cell destruction. Thus, in this model, the presence of LH resulted in increased proliferation of trophozoites in vitro and enhanced pathogenicity of N. fowleri in mice. However, neither in vitro cytotoxicity mechanisms nor the presence of membrane vesicles on the surface correlated with the pathologic potential of the amoeba. This indicated that the pathogenicity of N. fowleri remains a complex interaction between as-yet-unidentified cellular mechanisms.

INTRODUCTION

Species in the genus Naegleria are free-living amoebae found globally in soil and warm pools of fresh water (de Jonckheere & Voorde, 1977). About 30 species have been recognized, but only Naegleria fowleri is a human pathogen that causes primary amoebic meningoencephalitis (PAM) in immunocompetent children and young adults (De Jonckheere, 2004). Infection occurs during swimming in contaminated water or by inhalation of contaminated dust when the amoeba reaches the central nervous system through a nasal route along the olfactory nerve tracts (Carter, 1970). Several days after infection, a severe inflammation of the brain and meninges occurs, associated with haemorrhage and tissue necrosis (Jarolim et al., 2000). The patients suffer from a sudden severe headache, fever, vomiting, nausea and behavioural abnormalities, and most of them die within 1–2 weeks of the onset of symptoms (Carter, 1970). The ability of N. fowleri to induce such a fast progressing and fatal infection has underlined the need for an accurate understanding of the factors accounting for the pathogenicity of the amoeba.

Both in vivo mouse models (John & Hoppe, 1990; Martinez et al., 1971) as well as in vitro co-culture systems employing a broad range of different target cells have been used to study the pathogenesis of PAM (Fulford & Marciano-Cabral,
1986). It is commonly accepted that the pathogenicity of *N. fowleri* can be increased by serial mouse brain passages (John & Howard, 1993; Marciano-Cabral, 1988) as well as by passages in tissue cultures (John & John, 1989). In vitro, it has been shown that trophozoites destroy their target cells by a combination of phagocytosis and the cytolytic action of secreted enzymes (Visvesvara & Callaway, 1974). Brown (1978, 1979) suggests that *N. fowleri* injures target cells by piecemeal ingestion of cells, a process he called ‘trogocytosis’, using ‘food-cup’ structures on its surface (Fulford & Marciano-Cabral, 1986). Furthermore, a diverse array of proteins has been associated with *Naegleria*–mediated cell lysis (Marciano-Cabral & Cabral, 2007). Two pore-forming proteins, termed naegleriapores A and B, were isolated and lysin activity (Sohn et al., 2007) and gene silencing by small interfering RNAs targeting the *Nf*-actin gene (Jung et al., 2009) also suggested that *Nf*-actin is involved in *in vitro* cytotoxicity. In addition to *Nf*-actin, it has been reported that *N. fowleri* secretes proteases such as a cysteine protease, which catalyses the *in vitro* degradation of extracellular matrix proteins and exerts a cytotoxic potential against mammalian cells (Aldape et al., 1994).

Gene expression approaches and protein profiling to discriminate between highly pathogenic strains passaged in mice and axenically cultivated *N. fowleri* with lower pathogenicity identified changes in expression levels of selected genes. It was found that gene expression of virulence-related proteins was increased in highly pathogenic *N. fowleri* trophozoites, and in trophozoites co-cultured with mammalian target cells (Hu et al., 1991, 1992).

However, despite the many attempts to identify the determinants of pathogenicity, the cellular mechanisms and factors accounting for the destructive nature of PAM induced by *N. fowleri* are still unknown. In this study, a model was developed that allows modulation of the *in vivo* pathogenicity of the amoebo by different axenic culture conditions. Growth behaviour, morphology, *in vitro* cytotoxicity as well as transcriptional differences of selected genes were assessed as possible determinants of the pathogenic potential of *N. fowleri*.

**METHODS**

**In vitro cultivation of *N. fowleri***. Trophozoites of *N. fowleri* (ATCC 30863) were cultivated unagitated at 37 °C in 5 ml buffered PYNFH medium containing 1 % (w/v) Bacto peptone (BD Biosciences), 1 % (w/v) yeast extract (BD Biosciences), 0.1 % (w/v) yeast ribonucleic acid (Sigma), 15 mg folic acid (Sigma) L-1 and 1 mg haemin (Sigma) L-1 supplemented with 10 % (v/v) fetal calf serum (in 133 mM KH₂PO₄, 176.1 mM Na₂HPO₄) in Nunclon Δ Surface tubes (Fisher Scientific) from frozen stocks. In order to evaluate the influence of different culture conditions on the biological properties of *N. fowleri*, trophozoites were transferred to Nelson’s medium containing 0.1 % (w/v) liver hydrolysate (LH) (Sigma), 0.1 % (w/v) D- (+)-glucose (Sigma) and supplemented with 10 % (v/v) fetal calf serum in Page’s amoeba saline (2 mM NaCl, 16 μM MgSO₄, 27.2 μM CaCl₂, 1 mM Na₂HPO₄, 1 mM KH₂PO₄). A third medium composed of PYNFH medium supplemented with 0.1 % (w/v) LH allowed the evaluation of the influence of LH on biological properties of the amoebo.

To determine the proliferation rate, different media outlined above were inoculated with 2 × 10⁵ trophozoites per tube in 5 ml medium. After 24, 48, 72 and 96 h of cultivation, trophozoites were counted in three independent visual fields with an area of 1 mm² each (Nikon Eclipse TS 100). Values indicated are the means ± SEM of three experiments.

**Light microscopy (LM) and scanning electron microscopy (SEM) of *N. fowleri* trophozoites**. Trophozoites (10⁵ in 1 ml medium) were allowed to settle onto sterilized glass coverslips (Menzel-Glaser) placed in 24-well plates. After an incubation period of 1 h at 37 °C, the amoebea were fixed in 100 mM sodium cacodylate buffer (pH 7.2) containing 2.5 % glutaraldehyde (Sigma) for 1 h at room temperature. The fixed specimens were assessed by LM using an inverted microscope (Olympus IX51). For SEM, the trophozoites were subsequently post-fixed in 2 % O₃O₄ (Sigma) in cacodylate buffer for 2 h at room temperature. After extensive washing in distilled water, they were dehydrated by sequential washing in 30, 50, 70 and 100 % ethanol at room temperature, and were finally immersed twice in hexamethyldisilazene (Sigma) and air-dried. The coverslips were mounted onto metal stubs, sputter-coated with gold, and inspected on a JEOI 840 scanning electron microscope operating at 25 kV.

**Infection of mice with *N. fowleri***. In order to evaluate the pathogenicity of *N. fowleri* trophozoites grown in the different media described above, the C57BL/6 mouse model was used (Gianinazzi et al., 2005). Mice were purchased from Charles River and had access to food and water *ad libitum*. Animal experiments were approved by the Animal Care and Experimentation Committee of the Canton of Bern, Switzerland. Four-week-old female mice (n=36) were separated into experimental groups with 3–4 animals each. Animals were briefly anaesthetized by controlled inhalation of isoflurane, and intranasally infected by pipetting 2.5 × 10⁶ trophozoites into each nasal cavity. Trophozoites were maintained axenically for 2 weeks, finally grown to the late exponential phase in one of the three different media: PYNFH medium (n=15), Nelson’s medium (n=11) or PYNFH medium supplemented with 0.1 % LH (n=6). As controls, 10 μl PBS free of any *N. fowleri* was administered to four mice. All mice were clinically assessed by weight measurements and by documentation of symptoms characteristic for PAM such as ataxia, lethargy or any pain signs. At 2 weeks post-infection (p.i.) or at the time point when clinical signs of infection became apparent, the mice were sacrificed by CO₂ euthanasia and their brains were dissected. Brain tissue was investigated for the presence of viable trophozoites by taking a swab and cultivating at 37 °C in PYNFH medium with 100 μg penicillin and streptomycin (Gibco) ml⁻¹ to inhibit bacterial contaminations. The left hemispheres were stored in RNAlater (Ambion Europe) for 1 day at 4 °C and subsequently at −80 °C until isolation of DNA; the right hemispheres were fixed in 4 % formaldehyde in PBS for a maximum of 2 weeks at 4 °C.

**Histopathology**. To assess brain damage caused by *N. fowleri* infection and to localize the pathogen, brain tissue samples were analysed histologically according to Gianinazzi et al. (2005). For this...
In vitro cytotoxicity. In order to investigate the cytotoxic potential of N. fowleri, trophozoites were co-cultured with the murine fibroblast cell line L929 (ATCC 2148) in a contact-dependent and -independent system. For this purpose, 5 × 10^5 L929 cells per well were grown to confluence in 24-well plates in RPMI medium (Gibco, 0.5 ml per well) supplemented with 2% (v/v) GlutaMAX (Gibco), 10% (v/v) fetal calf serum and 100 μg penicillin and streptomycin ml^{-1} at 37 °C in a 5% CO2-enriched atmosphere.

For the contact-dependent cytotoxicity assay, L929 cells were washed three times with RPMI medium without supplement. Trophozoites to be evaluated were washed and resuspended in 100 μl RPMI medium. A volume corresponding to 5 × 10^5 trophozoites was added directly to the L929 cells in 0.5 ml RPMI medium.

For the contact-independent cytotoxicity assay, L929 cells and N. fowleri trophozoites were washed with PBS instead of RPMI medium, and trophozoites were resuspended in 100 μl PBS. Trophozoites (5 × 10^5) were added to cell culture inserts (Vitaxis) with a membrane pore size of 0.4 μm placed in the 24-well plate containing 0.5 ml PBS.

After 2, 6, 12 and 24 h co-cultivation at 37 °C, the cultures were inspected under an inverted microscope (Nikon Eclipse TS 100). At the same time points, the supernatant containing lactate dehydrogenase (LDH) released from damaged cells was collected and assayed by using the Cytotoxicity Detection kitPLUS (Roche). For negative and positive controls, L929 cells were cultivated in absence of N. fowleri, and positive controls were further processed by performing cell lysis through incubation in 20 μl lysis solution (Cytotoxicity Detection kitPLUS) at 37 °C for 15 min. As background control, RPMI medium without supplement for the contact-dependent assay or PBS for the contact-independent assay was used. For the cytotoxicity detection assay, 100 μl supernatant was transferred to a 96-well plate (performed in triplicate). After a 10 min incubation step in presence of 100 μl catalyst–dye solution in the dark at room temperature, 50 μl stop solution (both Cytotoxicity Detection kitPLUS) was added to the supernatants. The colour reactions were then quantified at 490 nm by using an ELISA reader (Molecular Devices). The cytotoxicity was calculated according to the following formula: cytotoxicity (%) = [(reaction value–background)–(negative control–background)]/[(positive control–background)–(negative control–background)] × 100. Values are means ± SEM of three experiments. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software).

RNA isolation and real-time PCR. In order to assess transcriptional differences between N. fowleri trophozoites grown in the different media, a quantitative real-time RT-PCR using primers against potential pathogenicity factors (Aldape et al., 1994; Herbst et al., 2002, 2004; Hu et al., 1991, 1992; Jung et al., 2009; Lee et al., 2007; Sohn et al., 2010) was performed. The primers used for the PCR are listed in Table 1. Briefly, RNA was isolated from approximately 10^7 trophozoites using the EZ1 RNA universal tissue kit (Qiagen) and EZ1 BioRobot (Qiagen). Trophozoites were resuspended in 750 μl QIAzo lysis reagent (Qiagen), and disruption and homogenization was done by using the TissueLyser operating for 3 min at 25 Hz. After incubation for 5 min at room temperature, 150 μl chloroform (Grogg) was added to the homogenized samples. A centrifugation step for 15 min at 12 000 g and 4 °C resulted in the separation of the sample into three phases; the aqueous phase was used as starting material for RNA isolation by the EZ1 BioRobot according to the manufacturer’s protocol. Quantification of RNA was performed by using the NanoDrop device. Real-time PCR was carried out using the QuantiFast SYBR Green RT-PCR kit (Qiagen) with a starting amount of 250 pg RNA per reaction on a Mastercycler ep realplex (Eppendorf). All reactions were performed in triplicate. Expression levels of the genes were given as values in arbitrary units relative to the amount of constitutively expressed 18S rDNA. Statistical analysis was done using GraphPad Prism 4.

RESULTS

Effects of medium composition on the axenic proliferation of N. fowleri trophozoites

Trophozoites exhibited different proliferation characteristics depending on the medium used for in vitro culture (Fig. 1). In all media, the maximum cell density was reached after an incubation time of 72 h. Trophozoites cultivated in Nelson’s medium reached a maximum cell density of about 1300 trophozoites per mm². While N. fowleri grown in PYNFH medium supplemented with LH gained an intermediate cell density of about 1000 trophozoites per mm², trophozoites in

Table 1. Primer sequences of potential pathogenicity factors

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Forward primer (5'–3')</th>
<th>Reverse primer (5'–3')</th>
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<tbody>
<tr>
<td>Nf-actin</td>
<td>AACTCAACAACACAACCTCCCTCCTCCCA</td>
<td>CCTTACACATACACGATCCGTTATCA</td>
</tr>
<tr>
<td>Calcineurin B</td>
<td>CCGAAGAGCCAATCATACAC</td>
<td>CAAGAACAGATATCCTCCAAAAGA</td>
</tr>
<tr>
<td>Naegleriapure A</td>
<td>TACGGAACCGAGCTGACAGTGA</td>
<td>TTGATTACCTGTGGCAACAATGG</td>
</tr>
<tr>
<td>Naegleriapure B</td>
<td>TTCCACACCGCTGATTAATACATG</td>
<td>GTAGCCACACCATTCACAAA</td>
</tr>
<tr>
<td>Virulence-related protein</td>
<td>CATGGTGGATGGAGTAAGAGTTG</td>
<td>TTTCTTCTCATTCTAGCTTCTT</td>
</tr>
<tr>
<td>Cysteine protease</td>
<td>TTACACTCCAGAGGAGCCGCGAG</td>
<td>GAAGGGTTGAGGAGTAGTTG</td>
</tr>
<tr>
<td>18S rDNA (housekeeper)</td>
<td>CATGTGACCACTAAGGCGGCAAG</td>
<td>CGGACGTTATCTAATGCTCAG</td>
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</table>


Effects of medium composition on the morphology of *N. fowleri* trophozoites

Culture conditions had an impact on morphology of *N. fowleri* as assessed by LM and SEM (Fig. 2). While the diameter of trophozoites grown in PYNFH medium was variable (between ~15 and 30 μm), trophozoites in both Nelson’s medium and PYNFH medium supplemented with LH were clearly smaller, exhibiting a reduced diameter of approximately 15 μm on average. In addition, SEM demonstrated that an increase in the number of membrane vesicles located on the surface of *N. fowleri* was a characteristic feature of the trophozoites cultivated in Nelson’s medium (Fig. 2f).

Impact of medium composition on *in vivo* pathogenicity of *N. fowleri*

From days 1 to 4 p.i., the weight of the mice increased by 0.88 ± 0.30 g per day. Between days 4 and 7 p.i., the mice infected with trophozoites grown in Nelson’s medium started to lose weight (Table 2), but they did not show any additional clinical signs. One day later, the animals became severely ill, showing pain symptoms (piloerection, hunching, closed eyes, tremor, stilting), kinetic ataxia, marked lethargy and ambulatory problems. This indicated that culture in Nelson’s medium had resulted in the generation of highly pathogenic *N. fowleri* causing symptoms characteristic of PAM in mice. The mortality rate of infected

PYNFH medium showed the lowest cell density of less than 600 trophozoites per mm². After 72 h of cultivation, all cultures had reached the stationary phase.

**Fig. 1.** Growth of *N. fowleri* trophozoites in different culture media. Nelson’s medium (●) produced rapidly proliferating trophozoites with a generation time of 1.84 ± 0.14 h (calculated in the exponential phase) and a maximum cell density of 1280 ± 80 trophozoites per mm². In contrast, trophozoites in PYNFH medium (◆) exhibited slower proliferation with a generation time of 3.98 ± 0.89 h and a maximum density of 578 ± 47 trophozoites per mm². *N. fowleri* in PYNFH medium supplemented with LH (■) had an intermediate maximum cell density of 1060 ± 87 trophozoites per mm² and a generation time of 1.70 ± 0.32 h. Values are means ± SEM of three experiments.

**Fig. 2.** Morphological effects of media composition on *N. fowleri* trophozoites. LM (a–c) and SEM (d–f) of *N. fowleri* grown in PYNFH medium (a and d), PYNFH medium supplemented with LH (b and e), and Nelson’s medium (c and f) revealed differences in cell diameter and surface structures. Trophozoites in PYNFH medium had a larger diameter (approx. 15–30 μm) than trophozoites in Nelson’s medium or in PYNFH medium supplemented with LH (approx. 15 μm). Membrane vesicles (f, indicated by arrows) were a characteristic feature of trophozoites maintained in Nelson’s medium. Bars, 200 μm (a–c); 10 μm (d–f).
animals was 100%, as mice had to be euthanized upon demonstration of the clinical signs listed above.

In contrast, 13 of 15 mice infected with trophozoites cultivated in PYNFH medium neither lost weight nor showed any clinical signs until the day of sacrifice. The remaining two mice showed symptoms characteristic for PAM, lost weight and died at day 6 p.i. (upon euthanasia). These results provided evidence that culture of *N. fowleri* in PYNFH medium resulted in amoebae of lower pathogenicity. The majority (86.7%) of mice infected with these amoebae survived the challenge.

Infection of mice with trophozoites grown in PYNFH medium supplemented with LH resulted in weight loss in the last 24 h before onset of symptoms between days 5 and 6 p.i. None of the mice challenged with these amoebae survived the infection, indicating that the addition of LH in PYNFH medium induced the generation of amoebae that were highly pathogenic in mice.

Thus, trophozoites grown in Nelson’s medium or in PYNFH medium supplemented with LH are referred to as highly pathogenic trophozoites, and those maintained in PYNFH medium are referred to as weakly pathogenic.

As shown by histopathology in animals presenting clinical signs, the presence of bleeding in the cortex surrounded by infiltrations of inflammatory cells indicated a severe inflammatory response (Fig. 3). High numbers of trophozoites were detected in the olfactory bulb as well as in the cortex of these animals. In contrast, in mock-infected mice and in mice without symptoms, the pathogen was not detected in brain tissue and there was no sign of any inflammatory response.

PCR analysis of brain tissue resulted in an *N. fowleri*-specific DNA amplification for mice with clinical signs, indicating a successful colonization of the brain by trophozoites. From the same brain tissues, trophozoites were successfully isolated and recultivated in PYNFH medium.

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**Table 2. Parameters of animal experiments**

Death represents the mean time after infection that mice from the individual experimental groups were euthanized due to severe clinical symptoms characteristic for PAM. ND, Not determined. Weight loss and death values are mean ± SD.

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>No. of mice</th>
<th>Infection dose</th>
<th>Weight loss (g per 24 h)</th>
<th>Death (days p.i.)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYNFH</td>
<td>15</td>
<td>5 × 10⁵ trophozoites</td>
<td>1.99 ± 0.04*</td>
<td>14 ± 0/6 ± 0*</td>
<td>13.3</td>
</tr>
<tr>
<td>Nelson</td>
<td>11</td>
<td>5 × 10⁵ trophozoites</td>
<td>2.49 ± 0.37</td>
<td>6.09 ± 0.83</td>
<td>100</td>
</tr>
<tr>
<td>PYNFH + LH</td>
<td>6</td>
<td>5 × 10⁵ trophozoites</td>
<td>2.37 ± 0.45</td>
<td>5.33 ± 0.52</td>
<td>100</td>
</tr>
<tr>
<td>PBS</td>
<td>4</td>
<td>10 µl</td>
<td>ND</td>
<td>14</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values belong to the 13.3% of mice that showed symptoms characteristic for PAM.

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**Fig. 3.** Histopathological assessment (haematoxylin–eosin) of mouse brains infected with *N. fowleri* (b and d) and of normal brain parenchyma of mock-infected mice and mice without clinical symptoms (a and c). The bleeding (indicated by an arrow in b) surrounded by inflammatory cells (intensely stained cells in d) is indicative of a severe inflammatory response in the brain of mice showing symptoms characteristic for PAM. In the same tissue, *N. fowleri* trophozoites were detected in high numbers in the olfactory bulb as well as in the cortex (insert in d). Bars, 300 µm (a) and (b); 100 µm (c) and (d); 10 µm insert.
Mock-infected mice and mice without symptoms revealed a negative PCR result, confirming the absence of trophozoites in brain tissue, as assessed by histopathology.

**Impact of medium composition on in vitro cytotoxicity of *N. fowleri***

In order to assess the cytotoxic potential of trophozoites in Nelson’s medium, PYNFH medium and PYNFH medium supplemented with LH, a lactate dehydrogenase release assay was performed (Fig. 4). Destruction of target L929 mouse fibroblasts occurred in a time-dependent manner over a 24 h period of co-cultivation with trophozoites. The monolayer of the target cells grown in the absence of *N. fowleri* (negative control) was still intact after 24 h of cultivation, i.e. a spontaneous lysis of L929 cells due to nutrition deprivation could be excluded.

Using a contact-dependent system (see Methods), weakly pathogenic trophozoites in PYNFH medium exhibited a significantly higher cytotoxicity than highly pathogenic trophozoites in Nelson’s medium at 12 and 24 h co-cultivation with target cells. Trophozoites maintained in PYNFH medium supplemented with LH showed a significantly higher cytotoxic behaviour against L929 cells than did trophozoites cultured in Nelson’s medium at 12 h, even though both were highly pathogenic for mice. This result indicated that the level of pathogenicity of *N. fowleri* did not correlate with the contact-dependent cytotoxic potential of the amoeba.

To further investigate the cytotoxicity of *N. fowleri*, a contact-independent system was used (see Methods). Here, the cytotoxic activity mediated by enzymes released into the medium was assessed. As particularly observed after 24 h co-cultivation, highly pathogenic trophozoites in Nelson’s medium exhibited a significantly higher cytotoxicity for target cells than weakly pathogenic trophozoites. At the same time point, the cytotoxicity of highly pathogenic *N. fowleri* in PYNFH supplemented with LH remained at low levels, indicating that the pathogenicity of the amoeba in vivo did not correlate with the level of contact-independent cytotoxicity in vitro.

**Gene expression patterns from potential pathogenicity factors of *N. fowleri***

In order to identify genes putatively involved in the modulation of the pathogenicity of *N. fowleri*, the transcription levels of previously described potential pathogenicity factors (see Introduction) were assessed by quantitative real-time RT-PCR (Fig. 5). Naegleriapore B and cysteine protease genes did not exhibit significant differences in transcription between trophozoites grown in the different media. However, differences in transcript levels were found for *Nf*-actin, calcineurin B, naegleriapore A and virulence-related protein genes. In highly pathogenic trophozoites grown in Nelson’s medium, *Nf*-actin and calcineurin B gene transcription was significantly upregulated compared with both weakly pathogenic trophozoites maintained in PYNFH medium and highly pathogenic trophozoites in PYNFH medium supplemented with LH. Since upregulation of *Nf*-actin and calcineurin B was not observed in the highly pathogenic phenotype of *N. fowleri* grown in PYNFH medium with LH, these two proteins were excluded as candidates for pathogenicity factors in our system. Transcript levels of the naegleriapore A gene was significantly higher in *N. fowleri* cultivated in PYNFH medium supplemented with LH than in trophozoites grown in the other two media. In summary, none of the potential pathogenicity factors used for PCR analysis could

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**Fig. 4.** In vitro cytotoxicity of *N. fowleri* trophozoites co-cultivated with L929 mouse fibroblasts. Cytotoxicity of weakly pathogenic trophozoites in PYNFH medium (white bars), and highly pathogenic trophozoites in Nelson’s medium (black bars) and in PYNFH medium supplemented with LH (grey bars) was assessed in a contact-dependent (a) and a contact-independent (b) assay. Values are means ± SEM of three experiments. Statistical analysis was performed using GraphPad Prism 4. Significant upregulation of cytotoxicity at some time points of co-cultivation was seen, as indicated by asterisks: *, 0.01 < *P* < 0.05; **, 0.001 < *P* < 0.01.
be attributed to in vivo pathogenicity in our experimental model. In contrast, a negative correlation between the levels of virulence-related protein gene transcription and pathogenicity was observed.

**DISCUSSION**

Although many attempts have been made to identify the factors responsible for the destructive nature of *N. fowleri* during PAM, the determinants responsible for the pathogenicity of *N. fowleri* are largely unknown. Several proteins, such as Nf-actin (Jung et al., 2009; Lee et al., 2007; Sohn et al., 2010), pore-forming naegleriapores A and B (Herbst et al., 2002, 2004), cysteine protease (Aldape et al., 1994) and virulence-related protein (Hu et al., 1991, 1992), have been implicated in Naegleria pathogenesis, but the exact roles of these proteins are not yet well understood. The present study enables modulation of the in vivo pathogenicity of *N. fowleri* by using different compositions of axenic growth media. As reviewed by Schuster (2002), *N. fowleri* has been adapted to several axenic media (Schuster, 2002), where of optimal growth was obtained with agitated cultures in Nelson’s medium (Haight & John, 1980). The same study showed a variation in cell yields among different *N. fowleri* isolates grown in Nelson’s medium (Haight & John, 1980). The isolate we used (ATCC 30863) achieved the maximum cell density of about 1300 trophozoites per mm² when maintained in Nelson’s medium. It appears that a simpler medium such as Nelson’s medium promotes better growth of *N. fowleri* than the more complex PYNFH medium, also consistent with the results of Haight & John (1980). When PYNFH medium was supplemented with LH, the growth of trophozoites improved, indicating that LH is a medium supplement promoting growth of the amoeba. Here, it was also striking that stimulation of cell proliferation by LH resulted in a massively reduced cell diameter. Furthermore, trophozoites cultivated in PYNFH medium supplemented with LH and Nelson’s medium, both containing LH, were highly pathogenic for mice, and trophozoites in PYNFH medium lacking LH were weakly pathogenic. These two findings suggest that LH is the determinant for both improved growth and elevated pathogenicity. This conclusion is in agreement with findings by Toney & Marciano-Cabral (1994a) demonstrating that Cline medium containing 0.55 % LH produces highly virulent amoebae (as compared with Nelson’s medium with 0.1 % LH) . However, a study by Bradley et al. (1996) showed that trophozoites (ATCC 30894) in Cline medium enriched with haemin were more pathogenic for B6C3F1 mice than those maintained in Nelson’s medium containing no haemin. This observation indicated that haemin in the culture medium enhances the pathogenic potential of the amoeba. In our experiments, haemin was also added to one of the culture media (PYNFH medium, used for growth of weakly pathogenic trophozoites) but at a concentration (0.0001 %) that was apparently not sufficient to induce pathogenicity of *N. fowleri* trophozoites. Apart from that, our high- versus low-pathogenicity model has to be regarded independently from that of Bradley and co-workers because different *N. fowleri* isolates (ATCC 30863 versus ATCC 30894) as well as different mouse strains (C57BL/6 versus B6C3F1) were used in the two infection models. Colonization, i.e. adherence of *N. fowleri* trophozoites to host cells is a critical step in inducing a successful infection (Marciano-Cabral & Cabral, 2007). Han et al. (2004) investigated the interaction of *N. fowleri* with extracellular matrix glycoproteins, such as fibronectin, located at the basal lamina and surrounding cells in tissue. In this study, a fibronectin-binding protein was identified, indicating that *N. fowleri* possesses a surface protein that is related to the human integrin-like receptor. Furthermore, Jamerson et al. (2012) showed a distinction between *N. fowleri* and non-pathogenic *Naegleria lovaniensis* in adhesion to and invasion of extracellular matrix components. Although this needs further investigation, missing surface proteins binding to fibronectin or to other extracellular matrix proteins may at least partially explain the inability of weakly pathogenic trophozoites to induce a successful colonization of the brain in our model.

According to Haggerty & John (1978), susceptibility to *N. fowleri* varies among different mouse strains with the complement-deficient strain (A/HeCr) being the most...
sensitive one. This observation indicates that innate immunity is an important factor in host defence against *N. fowleri* infection. Since PAM is a fast progressing disease, often fatal within 1–2 weeks p.i., the time to induce a specific and thus putatively effective immune response is limited. The fast progressing nature of the disease displays another argument for an important role of innate immunity as the first line of defence. The innate immune system may also be a factor accounting for the susceptibility of individual mice to *N. fowleri*. Thus, the 2 of 15 mice succumbing to infection with weakly pathogenic trophozoites may have failed to mount a response effective in combating invading amoebae, for unknown reasons.

Membrane vesicles located on the cell surface are a characteristic feature of *N. fowleri* maintained in Nelson’s medium (see Fig. 2f). Since trophozoites in Nelson’s medium showed a fast proliferation rate compared with trophozoites in the other two media (see Fig. 1), it can be excluded that vesicle formation was the consequence of an apoptotic process. Toney & Marciano-Cabral (1994b) showed that membrane vesicles are used to remove membrane-deposited proteins belonging to the membrane attack complex of the complement system, therefore serving as a mechanism to resist complement-mediated damage (Toney & Marciano-Cabral, 1994b). However, it remains questionable if this mechanism of complement inhibition contributes to pathogenesis, particularly because in our model, no obvious vesicle formation was found in those highly pathogenic *N. fowleri* trophozoites that had been cultivated in PYNFH medium supplemented with LH.

Although direct contact of amoebae to host cells must be considered to be a critical step in the pathogenesis of PAM (reviewed by Marciano-Cabral & Cabral, 2007), it was proposed that secretory-excretory metabolites released from amoebae exhibit cytotoxic effects on target cells (De Jonckheere, 1980), revealing a contact-independent mechanism of cytotoxicity. However, at least in our model, neither contact-dependent nor -independent cytotoxicity correlated with pathogenicity levels of the amoeba. This notion was supported by our finding that cytotoxicity of highly pathogenic trophozoites in PYNFH medium supplemented with LH was comparable to cytotoxicity of weakly pathogenic trophozoites maintained in PYNFH medium without LH supplement (see Fig. 4).

**Conclusion**

We showed that an increased concentration of LH in culture medium resulted in considerably smaller and more rapidly proliferating *N. fowleri* trophozoites with a higher pathogenic potential in the mouse model. The pathogenicity of *N. fowleri* most likely cannot be explained either by different cytotoxicity mechanisms, by the presence of membrane vesicles referring to complement resistance or a set of differentially regulated genes known as potential pathogenicity factors. In future, the *N. fowleri* infection model presented in our study may contribute to resolving the complex cellular and immunological mechanisms that determine pathogenicity of the amoeba.

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