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Influence of culture medium pH on internalization, growth and phenotypic plasticity of *Neospora caninum*

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ABSTRACT

Neospora caninum, a strictly intracellular protozoan, is a major leading cause of parasite-induced abortion in cattle. A widely held view of *N. caninum* infection is that both cellular proliferation and stage interconversion (tachyzoite–bradyzoite transformation) are triggered, perhaps even modulated by, changes in cultural conditions. This study tested the hypothesis that exposure of *N. caninum* tachyzoites to different pH culture media affects the parasite's entry, proliferation and cyst formation in cultured cells. The endocytic pathway for *N. caninum* entry into the K562 cell line was found to be mediated by low pH of culture medium. Internalization of *N. caninum* by host cells was significantly increased in acidic and alkaline culture medium compared to cells maintained in neutral medium as revealed by transmission electron microscopy. Parasite proliferation within Vero cells was assessed by plaque formation assay and was found to be highest when pH level was optimum, paralleled by a decrease in the number of cysts. In contrast, parasite encystation increased when the pH level was alkaline or acidic, as evaluated by indirect immunofluorescence and immunocytochemical analyses. Acidic pH regardless of state of host cell infection suppressed the rate of host cell division. These findings suggest that culture medium pH has a determinable effect on the host cell-*N. caninum* interaction and support the hypothesis that pH of culture medium influence the entry, growth, and phenotypic plasticity of *N. caninum* in mammalian cells.

1. Introduction

Neosporosis is an economically important disease of cattle and carnivores caused by the apicomplexan protozoan parasite *Neospora caninum* (Dubey et al., 2007). Effective prevention and treatment of neosporosis requires full understanding of the parameters that determine the entry, growth and survival of *N. caninum* in host cells. Infection by all apicomplexan parasites requires entry of the organism into the vertebrate host cell by attaching to and then creating an invagination in the host cell plasma membrane, and involves different protozoal ligands and cellular membrane receptors (Hemphill, 1996; Carruthers et al., 2000; Naguleswaran et al., 2002; Hemphill et al., 2006). Internalization and intracellular survival are critical mechanisms by which these organisms subvert the host's defence mechanism via providing the parasite with a protective niche against the host's immune response and against parasiticides that do not penetrate into host

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cells. Much of the knowledge regarding internalization and intracellular survival of apicomplexan protozoa has been gained from studies on *Toxoplasma gondii* (Botero-Kleiven et al., 2001; Robibaro et al., 2001). In contrast, very little is known about these critical events in *N. caninum* infection.

An intriguing feature of *N. caninum* and other cyst-forming apicomplexan protozoa is their autoreactivity: following exposure to stress, tachyzoite transforms from a growing state to a resting bradyzoite-containing cyst (Soete et al., 1994; Risco-Castillo et al., 2004; Hemphill et al., 2006; Ferreira da Silva Mda et al., 2008; Eastick and Elsheikha, 2010). This developmental differentiation is hypothesized to occur as an adaptive process allowing the parasite to cope with the adverse environments, and can be mediated or influenced by the complex cellular environment. Also, stage transformation process involves several paradoxical mechanisms that are meant for protection of the host but exploited by the parasite for its survival (Lyons et al., 2002; Elsheikha and Morsy, 2009).

Despite the large body of evidence available about the pathogenesis of neosporosis, the cellular mechanisms for *N. caninum* entry, growth and cyst formation are largely unexplored. Among the factors influencing the dimorphic switching of apicomplexan protozoa, pH of the culture medium is rarely studied in *N. caninum* (Soete et al., 1994; Weiss et al., 1995, 1999). In the present study, we investigate the hypothesis that the culture medium pH can govern the internalization, growth and cyst formation of *N. caninum*, knowledge which is needed to address the pathogenesis of a disease with significant economic implications.

2. Materials and methods

2.1. Cell lines and culture conditions

K562 cells, a human lymphoma cells were purchased from the Health Protection Agency Culture Collection (HPACC, Salisbury, Wiltshire, UK) and cultured in RPMI medium supplemented with 10% fetal calf serum (FCS) and antibiotic–antimycotic mixture. African Green Monkey kidney cell line (Vero) was purchased from the HPACC and used between passages 17 and 19. Cells were grown continuously as a monolayer in 75-cm^2 cell culture flasks in 15 ml of complete Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5–10% heat inactivated FCS (Invitrogen, GIBCO, UK), 25 mM HEPES, 2 mM L-glutamine, 10,000 U/ml penicillin G sodium, 10,000 μg/ml dihydrostreptomycin, 250 μg/ml amphotericin B, nonessential amino acids, and 100 mM sodium pyruvate. Cells were grown in the growth medium (10% FBS) at 37 °C in a humidified atmosphere containing 5% CO_2/95% air until the monolayer became confluent; then the medium was changed to maintenance medium (5% FCS). Stock cultures were maintained by lifting the monolayers with trypsin–EDTA (0.25% trypsin with 1 mM EDTA·4Na) once a week and transferring the cells to new culture flasks. To exclude if cell viability could be regarded as a factor effecting parasite invasion and therefore any subsequent induced interconversion, viability of cells was assessed using trypan blue exclusion assay prior to inoculation onto culture plates or flasks.

2.2. The parasite

N. caninum (Nc-Liverpool) strain was a gift from Professor Sandy Trees (University of Liverpool) and was used between passages 30 and 33. Parasite stock cultures were maintained in Vero cells for long periods by varying the FCS concentration. Parasites were harvested from their feeder cell culture and purified as described previously (Elsheikha et al., 2006). The number of tachyzoites was estimated using a haemocytometer. The final volume of suspension was adjusted so that 1×10^4 and 1×10^5 tachyzoites ml^{-1} of culture medium were used for the inoculation of Vero cells in 6-well plate and in culture flask assays, respectively. All experiments were conducted in triplicate.

2.3. Endocytosis assay

The effect of extracellular medium pH on the rate of endocytosis was determined on K562 cells infected with *N. caninum*, using FM2-10 dye. This dye has a fluorescence intensity that increases upon membrane incorporation. This property has been used to measure endocytosis, i.e. kinetic of plasma membrane internalization (Rauch and Farge, 2000). Therefore the kinetic of endocytosis can be obtained by measuring the increases in fluorescence intensity as a function of time. For this, approximately, 25×10^6 K562 cells/ml RPMI medium were infected with the parasite at a ratio of (1:1) and incubated at 37 °C for 2 h in media with acidic (6.5), neutral (7.5) and alkaline (8.5) pH. Different DMEM at pH values of 6.5 and 8.5 were made by using hydrochloric acid and sodium hydroxide, respectively. The cells were pelleted by centrifuging at 1000 rpm for 5 min, and the supernatant was removed. The cells were then washed once with sterile phosphate buffered saline (PBS). The cells were then centrifuged at 13,000 rpm for another 30 s. The supernatant was removed and the cell pellet resuspended in 1 ml of FM2-10 staining solution (FM2-10 final concentration 1 μM). After this 100 μl of the stained cells were transferred on a 96 well plate, and fluorescence (excitation 530 nm, emission 590 nm) was measured using the FLUROstar Optima BMG (Labtech, Germany). From the fluorescence intensity measured as a function of time, the kinetic of membrane internalization was deduced. In general, K562 cells display a kinetic of endocytosis in resting conditions of about 0.05%/s (Rauch and Farge, 2000).

2.4. Evaluation of N. caninum internalization

The rate of *N. caninum* internalization within K562 cells incubated in different pH culture media was assessed using transmission electron microscopy (TEM). K562 cells were infected with *N. caninum* and incubated in neutral, acidic, or alkaline medium for 2 days. Cells of each treatment were centrifuged and supernatants were discarded. The cell pellets were fixed overnight with 2.5% glutaraldehyde at 4 °C, post-fixed with 1% (w/v) OsO_4 for 1 h, dehydrated in increasing concentration of ethanol and embedded in Spurr's resin. Ultrathin sections were stained with 0.2% uranyl acetate and contrasted with lead citrate. The samples were observed using a JEM-1230 electron microscope (JOEL) and MegaView Software.