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The in vitro development of *Neospora caninum* bradyzoites

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Abstract

Neospora caninum is a recently identified apicomplexan protozoan parasite that is closely related to *Toxoplasma gondii*. *Neospora caninum* is of significant economic importance as it causes neurological disease and abortion in numerous animals. Antibodies to BAG1/hsp30 (also known as BAG5), a *T. gondii* bradyzoite-specific protein, have been demonstrated to react with *N. caninum* tissue cysts in vivo. Bradyzoite differentiation of *N. caninum* in vitro was investigated using culture conditions previously utilised for *T. gondii* in vitro bradyzoite development. Utilising the NC-Liverpool isolate of *N. caninum*, cyst-like structures developed within 3–4 days of culture of this parasite in human fibroblasts. In addition, an antigen reacting with mAb 74.1.8 (anti-BAG1) and rabbit anti-recombinant BAG1 was demonstrable by immunofluorescence, fluorescence-activated cell sorter, and immunoblot analyses. Expression of this antigen was increased by stress conditions, similar to that which has been described for *T. gondii* bradyzoite induction. Cyst-wall formation in vitro, as assayed by lectin binding, did not occur as readily for *N. caninum* as it does for *T. gondii*.

Keywords

BAG1; BAG5; Bradyzoite; Cyst; Differentiation; In vitro development; Lectin binding; *Neospora caninum*

1. Introduction

Neospora caninum is a recently discovered apicomplexan protozoan parasite that is closely related to *Toxoplasma gondii* [1]. This obligate intracellular organism causes neurological disease in many domestic and feral mammalian species [1, 2]. In addition, it is responsible for infectious abortions in cattle and other animals [1, 2]. These infections are of major economic importance. Dogs have been identified as definitive hosts for *N. caninum*, which suggests that human exposure to *N. caninum* may be common [3]. As yet, it is unclear if *N. caninum* is a human pathogen; however, serological data from humans are suggestive that infection may occur [4].

Transmission of *N. caninum*, as is true of *T. gondii*, can occur either by ingestion of oocysts, in faecally contaminated food or water, or via the ingestion of bradyzoites [3, 5, 6]. Several studies have demonstrated that *T. gondii* bradyzoites can develop in vitro and that the development of cyst-like structures in vitro can be demonstrated by TEM [6–11] as well as by bradyzoite-specific mAbs, such as mAb 74.1.8 [12–14]. The *T. gondii* bradyzoite-specific antigen BAG1/hsp30 (also known as BAG5) was cloned using mAb 74.1.8 [15]. Both mAb 74.1.8 and rabbit serum to this recombinant cloned protein (rabbit anti-rBAG1 [16]) reacted with *N. caninum* bradyzoites and cysts in vivo [16]. Neither of these antisera reacted with tachyzoites of *N. caninum* or *T. gondii* [16]. These reagents were, therefore, utilised to investigate the differentiation of tachyzoites to bradyzoites in *N. caninum* in vitro in human fibroblasts.

2. Methods

2.1. Culture of *Neospora caninum*

Neospora caninum NC-Liv [17] and NC-2 [18] isolates were maintained in human fibroblasts [ATCC CRL 1475 (CCD-27SK)]. Dulbecco's modified Eagle's Medium supplemented with 10% FCS (GIBCO-BRL), 10 mM Hepes (pH 7.1 or 8.1) and 1% penicillin–streptomycin was replaced weekly. Fibroblasts were subcultured weekly using 0.25% trypsin–0.03% EDTA at a subcultivation ratio of 1:4, and used between passages 6 and 30. Tylosin (Anti PPLO agent, GIBCO-BRL) was added to some cultures at a concentration of 60 $\mu\text{g ml}^{-1}$. ME49 and H7 (a BAG1 knockout [19]) strains of *T. gondii* were maintained by twice weekly passage in human fibroblasts as previously described [13]. In vivo cysts of *N. caninum* NC-Liv were purified from infected corticosteroid-treated mice as previously described [20].

2.2. Antibodies and lectins

Monoclonal antibody 74.1.8 (IgG2b, bradyzoite-specific reactive to a 28 kDa antigen BAG1/hsp30 aka BAG5 [21]) was used at 1:100 to 1:200 for immunofluorescence (IF) and 1:1000 for immunoblot analysis; polyclonal rabbit anti-recombinant BAG5 (BAG1/hsp30) [16] was used at 1:250 to 1:500 for IF and 1:1000 for immunoblot analysis. *Biotinylated Dolichos biflorans* lectin (Vector Laboratories) was used at a 1:200 dilution and streptavidin–Texas red (Vector Laboratories) was used at a 1:250 dilution for IF analysis.

2.3. In vitro immunofluorescence assay

Five-thousand *N. caninum* tachyzoites were used to infect a fibroblast monolayer in a two-chamber culture slide (Permanox, Nalge-Nunc). At the time of infection, pH adjusted media with or without tylosin was added. At 3 days p.i., the slides were washed in PBS (pH 7.2), fixed for 30 min with 2% buffered formalin, permeabilised with 0.2% Triton X-100 for 20 min, blocked with 1% BSA overnight. They were then incubated with the primary antibody(ies) at the appropriate dilution for 90 min at 37°C, washed three times in PBS, incubated with the secondary antibody 1:100 anti-mouse Texas Red–IgG or 1:200 anti-rabbit Texas Red–IgG (Southern Biotechnology), washed three times in PBS, overlaid with 2.5% DABCO (1,4-diazabicyclo-[2,2,2]octane)/PBS and examined with a Nikon Diaphot inverted fluorescent microscope. For lectin staining, the slide was incubated with 1:200 biotinylated *D. biflorans* lectin in 3% BSA/0.2% Triton X-100 for 30 min, washed three times with PBS, incubated with 1:250 dilution of streptavidin–Texas red in 3% BSA/0.2% Triton X-100 for 30 min, washed three times with PBS, overlaid with 2.5% DABCO/PBS and examined with a Nikon Diaphot inverted fluorescent microscope.

2.4. Immunoblot analysis

Organisms purified from human fibroblasts by rupture with a 27-gauge needle followed by filtration through a 3.0 μ M Nucleopore filter were split into equal samples, which were then assayed for protein concentration (BioRad) and dissolved in gel sample buffer as previously described [13]. Except where noted, equal amounts of protein and/or organisms (by counting of extracellular organisms in a haemocytometer) were loaded onto 10% SDS–PAGE gels, electrophoresed and transferred to nitrocellulose as previously described [13]. The amount of bradyzoite-specific antigen was ascertained by immunoblot analysis as previously described using mAb 74.1.8 [13]. Detection of bound antibody was performed using the Western Light Kit (Tropix), employing chemiluminescence with CSPD and an alkaline phosphatase-labelled secondary anti-mouse IgG antibody (1:10 000 dilution).

2.5. Flow cytometry

NC-Liv were harvested from infected human fibroblasts after 3–4 days of culture at pH 7.1, pH 8.1 or pH 7.1 with tylosin by lysis of the culture using a 27-gauge needle followed by filtration through a 3.0 μ m Nucleopore filter. The NC-Liv were then fixed for 30 min with 2% formalin, permeabilised with 0.2% Triton X-100 for 20 min, washed twice in PBS, incubated for 1 h with 1:250 rabbit polyclonal anti-rBAG1, washed twice in PBS, incubated for 30 min with 1:250 anti-rabbit fluorescein isothiocyanate IgG (Southern Biotechnology) and then washed twice in PBS. Flow cytometry was performed on a FACScan (Becton Dickinson Immunocytometry Systems). Logarithmic amplification was used for forward light scatter and side light scatter gating. The system threshold trigger was side scatter, and the threshold level was set so that system noise was below the trigger level. A dual-parameter light scatter gate was set on the predominant cluster of events. Logarithmic amplification was used for green fluorescein isothiocyanate (530 nm) fluorescence. An unstained suspension was used for scatter gating. Fluorescence background levels were determined using normal pre-immune rabbit serum as the primary antiserum. Ten-thousand cells were collected for each sample. All data were saved in List Mode format and analysed using CellQuest (Becton Dickinson Immunocytometry Systems).

2.6. Transmission electron microscopy

Cultures were fixed in 2.5% (v/v) glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.2) overnight at 4°C [22]. Following fixation, cells were rinsed in 0.1 M sodium cacodylate buffer, post-fixed in OsO₄, dehydrated in a graded ethanol series, placed in propylene oxide and embedded in Epon. Thin sections were placed on copper grids, stained with uranyl acetate/lead citrate and then examined with a Phillips JEOL TEM operated at 80 kV.

2.7. Immunogold electron microscopy

Fibroblasts, infected with *N. caninum*, were fixed in 0.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M cacodylate buffer for 1 h, rinsed in buffer, dehydrated through a graded ethanol series, embedded in LR white and polymerised for 48 h at 60°C. The tissue blocks were sectioned and sections placed on 300-mesh nickel grids coated with Formvar and carbon. Grids were incubated in blocking buffer [1% BSA–Fraction V (Sigma), 1% Tween 20 in PBS, pH 7.35 (PBST)] at 4°C for 1 h, followed by incubation with a 1:20 dilution of rabbit polyclonal anti-rBAG1 for 2 h at 20°C, followed by a 1:20 dilution of anti-rabbit IgG conjugated with 15 nm colloidal gold (Jackson Research). Grids were then washed five times in PBS, postfixed (10 min) in 1% glutaraldehyde, and washed in PBS [22]. Grids were then stained with 4% uranyl acetate/0.1% lead citrate and examined on a Phillips JEOL 1200 TEM.

3. Results

Previously, we had demonstrated by light microscopy, IF, TEM and immunoblot analysis the in vitro development of *T. gondii* ME49 strain bradyzoites and cysts in human fibroblasts [13]. Cyst-like structures with phase lucent cyst walls were observed in the current study in *N. caninum* NC-Liv and NC-2 cultures in human fibroblasts in vitro (Fig. 1A, B). These cyst-like structures were less frequent than the cysts seen with ME49 *T. gondii* in our previous studies using human fibroblasts [13]. Only one to two clearly defined structures with lucent cyst walls on phase contrast microscopy were observed for each slide culture. This suggests that, unlike *T. gondii*, the differentiation of *N. caninum* does not proceed as readily in human fibroblasts to completion. These *N. caninum* cysts were seen only in cultures containing tylosin (pH 7.1) (Fig. 1A) or in cultures maintained at pH 8.1 (Fig. 1B). This is analogous to observations on the development of *T. gondii* bradyzoites, where stress conditions such as pH 8.1, heat, or nitric oxide induce bradyzoite development and cyst formation [12–14, 23–25].

Antibodies (mAb74.1.8 [21] or polyclonal rabbit anti-rBAG1 [16]) to BAG1, a cytoplasmic *T. gondii* bradyzoite-specific antigen related to small heat-shock proteins, have been demonstrated to react with *N. caninum* bradyzoites in vivo (see Fig. 1D) [16]. No reactivity occurs with these sera with tachyzoites from either *N. caninum* or *T. gondii* in vitro or in vivo [13, 16]. Treatment of *N. caninum* NC-Liv cultures in human fibroblasts with either pH 8.1 or tylosin resulted in the formation of vacuoles containing BAG1 reactive parasites, suggesting that bradyzoite differentiation was occurring in vitro (Fig. 1C). The amount of induction varied considerably between experiments. Usually, 10–15% of vacuoles contained BAG1 reactive parasites under stress conditions. Similar results were obtained with *N. caninum* NC-2; however, the percentage of positive vacuoles was lower (usually less than 3% of all vacuoles).

The *T. gondii* cyst wall has been reported to contain carbohydrates and to react with various lectins [26] including *D. biflorans*. We have confirmed that ME49 *T. gondii* that are induced in vitro to form bradyzoites and cysts (Fig. 1J) also react with *D. biflorans* (Fig. 1I). In a similar fashion, rare *N. caninum* NC-Liv in tylosin media were demonstrated to display similar reactivity with lectins around the vacuole, suggesting formation of a cyst wall (Fig. 1G). More commonly, however, there was a patchy distribution of lectin staining around the vacuole (Fig. 1E), perhaps suggesting that cyst-wall formation had not been completed in these vacuoles (Fig. 1E).

To confirm that bradyzoite differentiation was occurring in vitro with *N. caninum* NC-Liv, fluorescence-activated cell sorter (FACS) analysis of these parasites cultured at pH 7.1, pH 8.1 and pH 7.1 with tylosin was employed (Fig. 2A–E shows a representative FACS analysis). Four independent experiments employing this FACS analysis demonstrated that $1.8 \pm 0.5\%$ (mean $\pm$ S.E.M.) of parasites at pH 7.1, $7.9 \pm 0.7\%$ of parasites at pH 8.1 and $21 \pm 4.5\%$ of parasites with tylosin at pH 7.1 reacted with rabbit anti-rBAG1. This confirmed that these conditions resulted in the induction of the *N. caninum* protein that reacted with the anti-BAG1 antiserum.

Immunoblot analysis was performed on *N. caninum* at pH 8.1 to characterise the *N. caninum* protein that reacted with rabbit anti-rBAG1. A 28 kDa reactive band identified in *N. caninum* was similar in size to the BAG1/hsp30 (BAG5) protein cloned from *T. gondii* [15, 27] (Fig. 3).

On TEM, vacuoles containing numerous parasites were observed. The parasites were found to contain typical apicomplexan structures, such as rhoptries, micronemes and dense granules [6]. Many parasites, especially at pH 8.1, contained large electron-lucent vacuoles

(presumably containing amylopectin) and subterminal nuclei, as is typical of bradyzoites (Fig. 4A) [2, 6]. In some of the parasitophorous vacuoles, the area between the parasites was electron lucent and contained a tubular network, as described for *T. gondii* [6]. The matrix of many parasitophorous vacuoles, however, was filled with a dense granular material. The granular matrix material was present throughout these vacuoles and the parasitophorous vacuolar membranes of such vacuoles were slightly thickened. Ten percent of the vacuoles at pH 8.1 reacted with rabbit anti-rBAG1 serum by immuno-EM. This reaction was localised primarily to the cytoplasm of the parasites (Fig. 4B). No staining was seen with control rabbit serum (data not shown). The cytoplasmic staining observed with *N. caninum* was similar to that which had previously been demonstrated to occur in *T. gondii* bradyzoites in vitro [13] and in vivo (Weiss, unpublished). Occasionally, reactivity was also seen in the matrix of *N. caninum* parasitophorous vacuoles with rabbit anti-rBAG1, which was not observed in *T. gondii*.

4. Discussion

We have demonstrated in vitro in human fibroblasts the expression of a bradyzoite-specific antigen of *N. caninum* by IF, TEM and immunoblot, as well as possible cyst formation. In *T. gondii*, stress conditions have been associated with the induction of bradyzoite development. It was found that temperature stress (43°C [24]), pH stress (pH 6.8 or 8.2 [13, 24]) or chemical stress (sodium arsenite [24]) resulted in an increase in bradyzoite antigen expression by *T. gondii* in culture, and an increase in the observed number of cyst-like structures. In murine macrophage lines derived from bone marrow, interferon- γ increased bradyzoite antigen expression, which appeared to be related to nitric oxide (NO) induction [23]. Similarly, when *T. gondii* was grown in host cells with a non-functional mitochondrial respiratory chain, both oligomycin (an inhibitor of mitochondrial ATP synthetase function) and antimycin A (an inhibitor of the electron transport of the respiratory chain) [23, 27] increased bradyzoite antigen expression, although not to the same extent as NO [23]. In *N. caninum* it also appears that stress conditions, i.e. pH 8.1, result in the induction of a bradyzoite specific antigen that appears to be a homologue of BAG1/hsp30 as well as ultrastructural changes consistent with bradyzoite differentiation. It is probable that similar mechanisms are involved in the transition between tachyzoites and bradyzoites in *Neospora* and *Toxoplasma*.

Immunoblot analysis demonstrated that under stress conditions, there was an induction of an antigen in *N. caninum* cultures reactive to rabbit anti-rBAG1. This antigen was 28 kDa, which is identical in size to BAG1/hsp30, which has been identified in *T. gondii* as a bradyzoite-specific cytoplasmic protein [15, 27]. In addition, immuno-EM demonstrated a similar cytoplasmic localisation for this immunoreactivity, in both *T. gondii* [13] and *N. caninum*. It is quite likely that the *N. caninum* protein reactive to rabbit anti-rBAG1 is a homologue of the *T. gondii* BAG1, and is also related to small heat-shock proteins.

Development of mature cysts of *T. gondii* occurs in vitro. These cysts have cyst walls which stain with mAb specific to the in vivo cyst wall (i.e. mAb 73.18 [6, 21]) as well as with the lectins *D. biflorus* and succinylated wheat-germ agglutinin (S-WGA) [26]. These lectins react with *N*-acetylgalactosamine and *N*-acetylglucosamine, respectively, and it has been suggested that chitin is a component of the cyst wall [26]. Although with a low frequency, a thick refractile cyst wall was demonstrated to develop in vitro with *N. caninum*. This occurred more commonly with NC-Liv than with the NC-2 isolate, and was not seen with the NC-1 isolate (data not shown). This is consistent with data in *T. gondii* where low virulence strains, i.e. high cyst forming strains in mice, such as ME49, have a higher spontaneous rate of cyst formation in culture than do virulent strains such as RH [6, 24]. Feeding experiments in cats have demonstrated that tissue culture derived cysts of *T. gondii*

are biologically identical to cysts obtained from animal tissues [6, 28]. Since the definitive host of *N. caninum* has been identified [3], the ability of the cyst-like structures observed in the *N. caninum* cultures to complete the life-cycle of this parasite can now be addressed.

Neospora caninum cultured under stress conditions occasionally demonstrated a cyst wall similar to that seen in *T. gondii* as detected by *D. biflorus* lectin staining. This suggests that mature cysts can be formed in human fibroblasts by *N. caninum*. More commonly, there was a patchy distribution of lectin staining, suggesting that fully mature cysts had not developed in human fibroblast cultures within the 3 day culture period. While a thick cyst wall is characteristic of mature *N. caninum* cysts, young cysts in vivo have thin cell walls [2]. Thus, it is possible that the development of thick cyst walls in vitro may require a longer period than the 3–4 days used for culture. One limitation of these in vitro systems is that the rate of replication of tachyzoites, which is greater than that of bradyzoites, enables tachyzoites to destroy the cell monolayer, thereby obscuring bradyzoite formation in long-term cultures. In *T. gondii*, inhibiting the rapid growth of tachyzoites, either by drugs (pyrimethamine [23]), cytokines (interferon [13, 23, 24]) or by frequent removal [10], gradually increases the percentage of bradyzoites in culture, consistent with their slower replication rate. These techniques may be of use in the optimisation of in vitro *N. caninum* cyst culture.

Additionally, while *T. gondii* forms cysts in many organs, *N. caninum* forms cysts almost exclusively in neural tissue [2]. In murine astrocytes, developing *T. gondii* cysts are surrounded by glial fibrillary acidic protein (i.e. intermediate filaments) [22]. This structure may stabilise the cyst in early development. It is thus possible that neural host cells may be necessary for efficient in vitro development of mature *N. caninum* cysts.

Tylosin is a macrolide antibiotic related to erythromycin [29]. The action of this class of drugs on the Apicomplexa is most likely due to effects on the plastid [30]. The plastid (e.g. apicoplast) is involved in fatty-acid metabolism and interacts in other pathways with the mitochondria of these organisms [31, 32]. Interference with these pathways by tylosin may trigger stress-induced differentiation to bradyzoites in *N. caninum*. Unfortunately, while prolonged growth of *N. caninum* in tylosin induces differentiation, it results in the death of these parasites. Death occurs after several cycles of growth and invasion, similar to that reported with clindamycin in *T. gondii* [33]. The plastid of *T. gondii* is believed to be the drug target of clindamycin [34]. Thus, while tylosin induced bradyzoite differentiation in *N. caninum*, it is unlikely to be useful for studies that require viable organisms.

Overall, these studies indicate that *N. caninum*, similar to *T. gondii*, will differentiate from the tachyzoite to the bradyzoite stage in vitro. Cysts can also be demonstrated to form in vitro; however, the maturity of these cysts remains to be tested. It appears likely that similar mechanisms, i.e. stress-related differentiation, underlie developmental transitions in both of these apicomplexan parasites and that this pathway may involve the plastid [35]. Further studies will optimise the in vitro developmental conditions for *N. caninum*, as well as characterise the BAG1 homologue in this parasite.

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Fig. 1.

Development of bradyzoites and cysts of *N. caninum*. (A) Phase contrast microscopy demonstrating thick-walled cyst of *N. caninum* in vitro, pH 7.1, with $60 \mu\text{g ml}^{-1}$ tylosin. (B) Phase contrast microscopy demonstrating thick-walled cyst of *N. caninum* in vitro, pH 8.1. (C) Rabbit anti-rBAG1 staining (Texas red anti-rabbit IgG) of in vitro bradyzoites of *N. caninum*, pH 8.1. Cytoplasmic staining is present, similar to that of ME49 *T. gondii* in (J). (D) Rabbit anti-rBAG1 staining (HRP anti-rabbit IgG [15]) of an in vivo cyst of *N. caninum* isolated from mouse brain [19]. (E) *Dolichos biflorans* lectin staining (Texas red–streptavidin) of an in vitro cyst of *N. caninum*. Note patchy distribution of stain. (F) Phase contrast microscopy of *N. caninum* vacuole from (E) (40× objective). (G) *Dolichos biflorans* lectin staining (Texas red–streptavidin) of an in vitro cyst of *N. caninum*. Note uniform labelling of vacuole membrane, similar to that of ME49 *T. gondii* in vitro cyst in (I). (H) Phase contrast microscopy of *N. caninum* vacuole from (G). (I) *Dolichos biflorans* lectin staining (Texas red–streptavidin) of an in vitro cyst of *T. gondii* ME49, pH 8.1. (J) Rabbit anti-rBAG1 staining (Texas red anti-rabbit IgG) of in vitro bradyzoites of *T. gondii* ME49, pH 8.1. (A–J) Scale bar = $25 \mu\text{m}$.

Fig. 2.

Representative fluorescence-activated cell sorter analysis of *N. caninum* BAG1 expression in vitro. Flow cytometry was performed on a FACScan. Logarithmic amplification was used for forward light scatter (FSC-H) and side light scatter gating (SSC-H). Logarithmic amplification was used for green fluorescein isothiocyanate (530 nm) fluorescence scale used for the Y axis in (B–E). (A) Unstained specimen used for scatter gating. A dual-parameter light scatter gate was set on the predominant cluster of events. The enclosed area represents the gated region used for analysis of subsequent fluorescence-activated cell sorter (10 000 events in this region are collected and equal the sum of the events in R2 plus R3). (B) Control (fluorescein isothiocyanate labelled secondary antibody). No fluorescence is evident in the R2 region (area of positive fluorescence). (C) *Neospora caninum*, pH 7.1, stained with anti-rBAG1. R2 (BAG1 fluorescence) was 0.7% of total events. (D) *Neospora caninum*, pH 8.1, stained with anti-rBAG1. R2 (BAG1 fluorescence) was 8.7% of total events. (E) *Neospora caninum*, pH 7.1, and 60 $\mu\text{g ml}^{-1}$ tylosin stained with anti-rBAG1. R2 (BAG1 fluorescence) was 18.1% of the total events.

Fig. 3.

Immunoblot analysis with anti-BAG1. Lane 1: *N. caninum*, pH 8.1. Lane 2: *T. gondii* ME49, pH 8.1. Lane 3: *T. gondii* H7 (BAG1 knockout [19]), pH 8.1. Equal amounts (of protein) of *N. caninum* and H7 and a 10-fold lower concentration of ME49 *T. gondii* were loaded onto the gel. As can be seen, reactivity with the anti-BAG1 serum is to a 28 kDa antigen in both *N. caninum* and *T. gondii*. Similar staining was observed with mAb 74.1.8 (data not shown).

Fig. 4.

Electron microscopy of *N. caninum* bradyzoite development in vitro. (A) Transmission EM of developing cyst. This demonstrates large electron-lucent vacuoles and subterminal nuclei, as is typical of bradyzoites. Scale bar = 0.5 μm. (B) Immuno-electron microscopy using rabbit anti-rBAG1 and a 15 nm gold secondary antibody. Staining (arrow) is present in the cytoplasm. The immunogold staining between the two parasites at the top of the figure, around the electron lucent vacuoles, is within another parasite that could be clearly seen on additional sections. Scale bar = 0.5 μm.