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NEOSPORA CANINUM IN VITRO: EVIDENCE THAT THE DESTINY OF A PARASITOPHOUS VACUOLE DEPENDS ON THE PHENOTYPE OF THE PROGENITOR ZOITE

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Abstract

We previously reported that *Neospora caninum* can be induced to express BAG1, a bradyzoite antigen, within 3 days of culture under stress conditions. The main goals of the present experiment were to increase the expression of BAG1 in vitro (in part by extending cultures for 9 days), to observe parasitophorous vacuoles at various points of stage differentiation, and to test the ability of organisms produced in vitro to function like mature bradyzoites. Expression of BAG1 and of a tachyzoite antigen (NcSAG1) was monitored using a double-label immunofluorescence assay. For the purpose of this study, organisms expressing NcSAG1 were designated as tachyzoites, those expressing BAG1 were designated as bradyzoites, and those expressing both antigens were designated as intermediate zoites. The greatest percentage of intermediate zoites and bradyzoites (14%) occurred in bovine monocytes maintained for 9 days. These bradyzoites did not appear to be functionally mature; they did not induce patent infections in dogs, in contrast to bradyzoites that were produced in chronically infected mice. In vitro, large parasitophorous vacuoles contained either a pure population of tachyzoites or a mixture of tachyzoites and intermediate zoites, which is indicative of asynchronous stage conversion of organisms within a vacuole. Bradyzoites were first observed within small vacuoles on day 6, and bradyzoites never shared vacuoles with tachyzoites. This finding suggests that vacuoles containing bradyzoites may develop only if the cell is invaded by a zoite that has already begun bradyzoite differentiation. An alternative possibility is that cysts may develop if the establishing tachyzoite undergoes bradyzoite differentiation before multiplying. Cysts do not appear to arise from transformation of tachyzoites within large parasitophorous vacuoles.

Infection with *Neospora caninum*, an apicomplexan protozoan, can cause abortions in cattle (Thilsted and Dubey, 1989) and goats (Barr et al., 1992). Dogs are a definitive host (McAllister et al., 1998), and ruminants are intermediate hosts. Wild canids and wild ruminants are suspected to be a natural reservoir for the infection (Dubey et al., 1999; Barling et al., 2000).

Two asexual stages of the parasite occur within the tissues of intermediate hosts. Tachyzoites replicate quickly within parasitophorous vacuoles, which eventually cause lysis of the host cell. The bradyzoite stage replicates slowly and forms intracellular tissue cysts. Tachyzoite to bradyzoite conversion allows the parasite to endure in a latent stage for an indefinite period of time. Unlike tachyzoites, bradyzoites are able to survive gastric

digestion (Lindsay et al., 1992) and induce patent infections in the definitive host by carnivorism.

Bradyzoites are needed for scientific investigation. First, bradyzoites are needed for investigation of the factors that induce recrudescence of latent infection. Recrudescence is responsible for vertical propagation of *N. caninum* within infected lines of cows (Björkman et al., 1996) and may also be a cause of systemic neosporosis in dogs. Second, a supply of bradyzoites could enable the development of an oral vaccine to prevent farm dogs from shedding *N. caninum* oocysts and thus infecting cattle.

We previously reported that *N. caninum* can be induced to express BAG1, a bradyzoite antigen, within 3 days of culture in human fibroblasts under stress conditions (Weiss et al., 1999). The main goals of the present experiment were to increase the proportion of organisms that express BAG1 in vitro, to observe expression of BAG1 and also of a tachyzoite antigen (SAG1) within parasitophorous vacuoles over a longer period (9 days), and to determine if the BAG1-expressing organisms have the ability to function like mature bradyzoites.

MATERIALS AND METHODS

Examination of individual organisms

Parasites—Tachyzoites of *N. caninum* (NC-Liverpool strain) (Barber et al., 1995) were maintained at 37 C and 5% CO₂ in human foreskin fibroblasts (American Type Culture Collection [ATCC], Manassas, Virginia) in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Tachyzoites were passaged twice weekly. Also, tissue cysts of *N. caninum* were produced in immunosuppressed mice as previously described (McGuire, McAllister, Jolley, and Anderson-Sprecher, 1997).

Cell cultures—Several in vitro culture protocols were investigated to determine which would produce the most organisms expressing BAG1 antigen. Application of *N. caninum* antiserum to culture media, use of feline astrocytes (ATCC), and incubation at 37 C did not perform well in preliminary experiments and, therefore, were excluded from further experiments. The protocols used in the present experiment are listed in Table I. Two different cell types were evaluated as host cells: human foreskin fibroblasts (ATCC) and bovine monocytes (M-617) (Speer et al., 1985). Before infection, cell cultures were grown in 6-well plastic tissue culture flasks (9.2 cm²/well; Costar, Cambridge, Massachusetts), the temperature was elevated to 41 C for 2 hr to adapt the host cells, and then the temperature was returned to 37 C. *Neospora caninum* tachyzoites were harvested from flasks of infected human foreskin fibroblasts during a period of rapid growth, and confluent monolayers within the wells were each infected with 550 tachyzoites/cm². The wells were maintained at 37 C for 24 hr to permit rapid growth of tachyzoites. Twenty-four hours after infection, the media in all culture systems were replaced with DMEM supplemented with 10 mM *N*-2-hydroxyethylpi-perazine-*N'*-2-ethane-sulfonic acid and 5% *N. caninum* seronegative bovine calf serum (obtained from a presuckle Jersey calf), the pH was adjusted to 8.1 (using NaOH), and the incubation temperature was raised to 41 C. In addition, media in some cultures were supplemented with 1 µg/ml heat shock protein 70 (Sigma Chemical Co., St. Louis, Missouri) or with 20 nM sodium nitroprusside (a source of NO, which also induces endogenous production of heat shock proteins in monocytes) (Sigma). Media were changed twice weekly.

Antibody reagents—The expression of BAG1, a small heat shock protein also known as BAG5, was detected using a rabbit-derived polyclonal antibody (McAllister et al., 1996). A

mouse-derived monoclonal antibody (6C11) was used to detect the expression of a tachyzoite surface antigen, NcSAG1 (Howe et al., 1998; Howe and Sibley, 1999). For this investigation, organisms expressing NcSAG1 were designated as tachyzoites, those expressing BAG1 were designated as bradyzoites, and those expressing both antigens were designated as intermediate zoites. Two secondary antibodies were used in the immunofluorescence assays described below: rhodamine-conjugated goat anti-rabbit IgG (Pierce, Rockford, Illinois) and fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Bethyl, Montgomery, Texas).

In vitro double-labeling immunofluorescence assay—Parasites from all cell culture protocols were harvested on days 3, 6, and 9 after treatment. Infected monolayers were scraped with a rubber policeman, washed in phosphate-buffered saline (PBS), and passed 5 times through a 26-gauge needle to disrupt cell membranes. The total number of organisms per well was estimated using a hemocytometer. The organisms were pelleted and resuspended in 20:1 of PBS. The entire suspension was smeared onto positively charged microslides (BioGenex, San Ramon, California). The slides were air dried, immediately fixed for 30 min in 2% paraformaldehyde with 0.1% Triton X-100 (Sigma), blocked with 1% bovine serum albumin for 20 min at room temperature, washed 3 times in PBS, incubated with a mixture of anti-BAG1 (1:250) and 6C11 (1:1,000) antibodies for 90 min at 37 C, washed 3 times in PBS, incubated with a mixture of the secondary antibodies for 90 min at 37 C, washed 3 times in PBS, mounted under glass coverslips using ProLong Antifade Kit (Molecular Probes, Eugene, Oregon), and examined with a Nikon Eclipse E 600 fluorescent microscope equipped with a Spot RT Slider digital camera. Two photomicrographs were taken for each field of view, one using an excitation wavelength between 460 and 500 nm with a band-pass emission filter between 510 and 560 nm and other using an excitation wavelength between 530 and 550 nm with a band-pass emission filter between 590 and 650 nm. Paired images were overlaid and examined visually to determine which organisms were labeled by one or both antibody reagents.

Statistical analysis—Four replicates were performed for each protocol. For each smear, a total of 400 organisms were counted in random fields. For each count, the numbers of single- and double-labeled organisms were recorded. Statistical comparisons of the protocols were performed using analysis of variance. Analyses were performed to identify protocols with both the highest proportion of bradyzoites, or bradyzoite plus intermediate zoites, and the highest estimated numbers of these organisms. Proportions were analyzed after an arcsine transformation, and numbers of organisms were analyzed after a log transformation. Post hoc analyses were based on Tukey's honestly significant difference to account for inflation of effect size from multiple comparisons (Kuehl, 2000).

Tests of bradyzoite function

Acid-pepsin digestion assay—For the purpose of this assay, protocol no. 9 (Table I) was repeated. The infected monolayers were scraped with a rubber policeman, and cells were disrupted by passing 5 times through a 26-gauge needle. The parasites were washed and resuspended in PBS, incubated for 5 min at 37 C with an equal volume of pepsin-HCl solution (0.5 g NaCl, 0.52 g pepsin, 1.3 ml concentrated HCl, and 98.7 ml water), and washed in PBS. This suspension was then inoculated onto a fresh monolayer of human foreskin fibroblasts maintained at 37 C, pH 7.2. Cells were examined daily for tachyzoites for 3 wk. Bradyzoites of *N. caninum* produced in mice are known to survive this procedure (Lindsay and Dubey, 1990; McGuire, McAllister, and Jolley, 1997). To rule out the possibility that the incubation time could kill organisms (rather than the acid-pepsin solution), a standard cell culture containing tachyzoites was disrupted, and organisms were suspended in PBS for 30 min before inoculation onto a fresh monolayer.

Bioassay in dogs—Four, 3- to 4-mo-old, mixed breed hound dogs were purchased from a class A breeder (Covance, Kalamazoo, Michigan). Before the experiment, the dogs were confirmed to be seronegative for *N. caninum* using a previously described indirect fluorescent antibody test (IFAT) procedure at a 1:25 dilution (Dubey et al., 1988). For 5 days before infection, fecal samples were obtained from each dog, floated in Sheather's sucrose solution, and examined microscopically for protozoal oocysts. Two dogs were each dosed orally with 150 cm² *N. caninum*-infected monolayers using culture protocol no. 9 and also with an additional 150 cm² cell culture using protocol no. 3 (Table I). On the basis of the results in Table I, each dog is expected to have ingested about 66,000 bradyzoites and an additional 99,000 intermediate zoites. The remaining 2 dogs were each fed 4 skinned mice with chronic neosporosis, using previously described methods (McGuire, McAllister, Jolley, and Anderson-Sprecher, 1997; McAllister et al., 1998). The dogs' feces were collected, floated, and examined daily for 3 wk, starting on day 4 after infection.

Gerbil bioassay—A rodent bioassay was used to seek evidence of the development of patent *N. caninum* infections in dogs, similar to previously reported methods (McAllister et al., 1998). The only significant modification was substitution of gerbils for mice because gerbils are more highly susceptible to oral inoculation with *N. caninum* than are mice (Dubey and Lindsay, 2000). For each dog on each day (beginning on day 4), the feces were mixed and a 2-g fecal sample was suspended in 2% H₂SO₄ and placed on a shaker for 7 days to permit sporulation of any oocysts that may have been present. Additional 2-g samples were added to the specimen jars daily for 7 days (total 14 g feces), and then another specimen jar was begun, for a total of 3 wk. Afterward, the suspensions were neutralized, centrifuged, and each week's specimen was mixed into rodent chow and fed to 2 gerbils (for a total of 3 wk or 6 gerbils/dog). Development of *N. caninum* infections in gerbils was assessed 1 mo after exposure using IFAT serology (1:50 dilution) and polymerase chain reaction (PCR) of brain.

Visual observation of intact parasitophorous vacuoles

Human foreskin fibroblasts were grown in 2-chamber tissue culture glass microslides (4.5 cm²/chamber: Rochester, New York). Monolayers were infected and stressed by elevation of heat and pH, as in protocol no. 3 (Table I). On days 3, 6, and 9 after treatment, entire slides containing infected monolayers were fixed in 2% paraformaldehyde for 30 min, washed 3 times in PBS, permeabilized with 0.2% Triton X-100 for 20 min, and then further processed for double-label immunofluorescence using BAG1 antiserum and 6C11 monoclonal antibody, as described above.

RESULTS

Examination of individual organisms

One goal of this investigation was to increase the proportion of organisms expressing BAG1 antigen in vitro. The mean number of organisms per 9.2-cm² well and the percentages of bradyzoites and intermediate zoites are shown in Table I. Previously reported values for bradyzoite production (Weiss et al., 1999) should be compared with bradyzoites plus intermediate zoites in the present experiment. The greatest percentages of bradyzoites (7.7%) and intermediate zoites (6.4%) (total 14.1%) occurred on day 9 in bovine monocytes, without the addition of sodium nitroprusside (superior to all other protocols, $P < 0.001$). This protocol also produced the highest concentration of bradyzoites in cell culture, 316/cm² (Fig. 1).

Tests of bradyzoite function

A second goal of this investigation was to test the bradyzoites produced in vitro to determine whether they possess functional characteristics of mature bradyzoites that occur in vivo. In vitro-produced bradyzoites and tachyzoites did not survive the 5-min incubations with acid-pepsin solution. Tachyzoites incubated extracellularly for 30 min in the absence of acid-pepsin solution remained viable, thereby demonstrating that time alone did not kill the organisms in the acid-pepsin digestion trial.

Neither of the 2 dogs produced oocysts after consuming infected tissue cultures, as determined by the examination of fecal flotations and a bioassay in gerbils (all 12 gerbils were negative by IFAT and PCR). In contrast, both dogs fed chronically infected mice shed oocysts in their feces beginning on days 11 and 17 after infection. In addition, the gerbils that consumed feces from the times when oocysts were observed became seropositive (6/12 gerbils), and the presence of *N. caninum* in the central nervous system was demonstrated in seropositive gerbils by PCR. Four weeks after exposure, dogs had IFAT titers of < 1:25 (a dog that consumed mice), 1:25 (the second dog that consumed mice), 1:50 (a dog that consumed cultured organisms), or 1:100 (the second dog that consumed cultured organisms).

Visual observation of intact parasitophorous vacuoles

Our third goal was to observe the developing parasitophorous vacuoles or early cysts at various time points, after they had been stained with surface markers for both tachyzoites and bradyzoites. Parasitophorous vacuoles that contained only tachyzoites predominated at all times and measured up to 100 µm in diameter (Fig. 2A). Also observed at all times were lesser numbers of vacuoles that contained a mixture of tachyzoites and intermediate zoites. This type of vacuole was also up to 100 µm in diameter (Fig. 2B). The number of intermediate zoites within these vacuoles varied from 1 or 2 to approximately 50% of the organisms. Some of these vacuoles had ruptured (Fig. 2C). Intracellular vacuoles that contained bradyzoites were not observed on day 3 but were present on days 6 and 9 (Fig. 2D). Although bradyzoites sometimes resided with intermediate zoites, they never shared the same parasitophorous vacuole with tachyzoites. Vacuoles containing bradyzoites were smaller than those containing tachyzoites, between 7 and 20 µm in diameter. The smallest vacuoles or cysts contained only 1, 2, or 4 bradyzoites. On day 9, phase contrast microscopy revealed a thickened wall around a few parasitophorous vacuoles, consistent with the development of a cyst wall (Fig. 3).

DISCUSSION

Our first goal was to increase the proportion of *N. caninum* bradyzoites produced in vitro. Gains were modest. The percentage of organisms expressing BAG1 increased with time. A greater percentage of bradyzoites were formed in slowly replicating bovine monocytes than in quickly replicating human foreskin fibroblasts. About 14% of organisms expressed BAG1 antigen by day 9 (protocol no. 9), but less than 8% of organisms expressed BAG1 without coexpression of the SAG1 tachyzoite antigen.

Our second goal was to determine whether in vitro-produced bradyzoites were functionally mature, but they did not appear to be. After 9 days of culture, the organisms did not survive acid-pepsin digestion and did not induce patent infections in either of the 2 dogs. In contrast, 5–7 days of cell culture of *Toxoplasma gondii* can produce bradyzoites that induce patent infections in cats (Hoff et al., 1977; Lindsay et al., 1991; Popiel et al., 1994). Longer incubation in vitro may be needed for the development of functionally mature *N. caninum* bradyzoites. This possibility is consistent with the observation that *N. caninum* tissue cysts

are seldom found earlier than 21 days after infection of mice (Lindsay and Dubey, 1989). Future attempts at in vitro induction of *N. caninum* bradyzoites should address the possibility of longer incubations and more effective elimination of rapidly dividing tachyzoites. Both dogs consuming cultured organisms developed low antibody titers, suggesting that tachyzoites may have penetrated oral or esophageal mucosa. One of the dogs that shed oocysts (after ingestion of infected mice) did not develop a detectable antibody titer, which has been observed previously (McAllister et al., 1998).

Our third goal was to make qualitative observations about the expression of bradyzoite (BAG1) and tachyzoite (SAG1) antigens at various time points, and the results were intriguing. Intermediate zoites were observed that coexpressed both these antigens, and parasitophorous vacuoles often contained a mixture of tachyzoites and intermediate zoites. It is generally assumed that apicomplexan organisms within a parasitophorous vacuole are clones derived from a single progenitor zoite (Bohne et al., 1993). Therefore, the finding of *N. caninum* tachyzoites and intermediate zoites within the same vacuole indicates that stage conversion can occur asynchronously within a cloned intravacuolar population. This is similar to findings with *T. gondii* (Bohne et al., 1993; Soete et al., 1993). These mixed vacuoles can rupture, as was observed in the present experiment, thereby providing an opportunity for intermediate zoites to spread to new cells.

Neospora caninum bradyzoites were first observed within small vacuoles on day 6, they were never observed to share vacuoles with tachyzoites, and the walls of the vacuoles containing bradyzoites sometimes appeared thick when observed by phase contrast microscopy. These observations suggest that cysts containing bradyzoites may develop only if the cell is invaded by an organism that has already begun differentiation. An alternative possibility is that cysts may develop if the establishing tachyzoite undergoes bradyzoite differentiation before multiplying. In either case, the fate of a vacuole to form a cyst appears to be determined as an early event that either precedes or immediately follows invasion. Cysts do not appear to arise from direct transformation of parasitophorous vacuoles containing clusters of tachyzoites.

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Figure 1.

Box-plot graph demonstrating the minimum, first quartile, mean, third quartile, and maximum concentration (organisms/cm²) of *Neospora caninum* bradyzoites or of combined bradyzoites and intermediate zoites, after 9 days of cell culture at 41 C and pH 8.1. Culture systems included human foreskin fibroblasts (HFF) with (+) or without 20 nM sodium nitroprusside or bovine monocytes (BM) with (+) or without 1 µg/ml heat shock protein 70. Bradyzoites expressed only BAG1 antigen, and intermediate zoites expressed both BAG1 and NcSAG1 antigens.

Figure 2.

Double-labeled intracellular *Neospora caninum* organisms. Organisms labeled only with anti-BAG1 are red (designated as bradyzoites). Organisms labeled only with anti-NcSAG1 are green (designated as tachyzoites). Double-labeled organisms appear in shades of orange and yellow (intermediate zoites). Bars = 10 μ m. **A.** Parasitophorous vacuole containing only tachyzoites. **B.** Parasitophorous vacuole containing a mixture of tachyzoites and intermediate zoites. **C.** Ruptured parasitophorous vacuole composed of approximately equal numbers of tachyzoites and intermediate zoites. **D.** Three small parasitophorous vacuoles composed of bradyzoites, a slightly larger parasitophorous vacuole containing a mixture of tachyzoites and intermediate zoites and 2 individual intermediate zoites.

Figure 3. Phase contrast illumination of intracellular *N. caninum* in vitro. Note the presence of a thickened wall (arrow) around a parasitophorous vacuole, consistent with formation of a cyst. Bar = 10 μ m.

Table 1

Protocols evaluated for in vitro production of *Neospora caninum* bradyzoites. All cell cultures were maintained at 41 °C and pH 8.1. The phenotype of the organisms was determined by a double-labeling immunofluorescence assay using antibodies against bradyzoite-specific (BAG1) and tachyzoite-specific (NcSAG1) antigens. Bradyzoites were labeled only with anti-BAG1. Intermediate zoites were labeled with both anti-BAG1 and anti-NcSAG1. Standard errors for mean percentage 0 are estimated using $P = 0.5/n = 0.0003$. Other standard errors are based on the 4 replicates per protocol.*

Protocol	Cell type and treatment	Time of harvest (days PI)	Mean number of harvested organisms	Mean % of bradyzoites (SE)	Mean % of bradyzoites plus intermediate zoites (SE)
1	HFF	3	682,500	0.00 (0.04)	0.06 (0.06)
2	HFF	6	990,000	0.00 (0.04)	1.44 (0.45)
3	HFF	9	155,000	0.75 (0.40)	3.12 (0.95)
4	HFF + HSP	3	412,500	0.00 (0.04)	0.00 (0.04)
5	HFF + HSP	6	910,000	0.19 (0.12)	1.44 (0.48)
6	HFF + HSP	9	142,500	0.62 (0.33)	2.12 (0.75)
7	BM	3	187,500	0.00 (0.04)	0.06 (0.06)
8	BM	6	95,000	0.94 (0.21)	2.75 (0.44)
9	BM	9	37,500	7.75 (1.43)	14.12 (1.89)
10	BM + SNP	3	155,000	0.00 (0.04)	0.44 (0.21)
11	BM + SNP	6	87,500	0.12 (0.07)	0.56 (0.24)
12	BM + SNP	9	9,500	3.25 (0.78)	9.69 (1.77)

* HFF, human foreskin fibroblasts; BM, bovine macrophages; HSP, heat shock protein 70 (1 µg/ml); SNP, sodium nitroprusside (20 nM); PI, postinfection.