Generation of Parasite Cysts in Cultured Cells Instead of Living Animals

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Summary
In order to develop any effective means of prevention or therapy against Neospora caninum infection, all three life cycle stages of this parasite have to be taken into account. In this project, the in vitro culture of N. caninum bradyzoites containing tissue cysts has been developed, thus allowing to the initiation of stage conversion without the use of animal experimentation. This enables researchers to study novel aspects of stage differentiation, to investigate novel diagnostic antigens, and explore the usefulness of potentially interesting drugs and drug targets not only in tachyzoites but also in bradyzoites.

Keywords: parasites, neosporosis, bradyzoites, Vero cells

Background Information

Neospora caninum and neosporosis

Neospora caninum (Apicomplexa: Eimeriina: Sarcocystidae) is a Toxoplasma-like parasite that was first identified in 1984 in dogs with encephalomyelitis and myositis. It was later also reported in various species of livestock, especially cattle. In the USA, the EU, and many other countries worldwide, neosporosis is the leading diagnosed cause of abortion in cattle, and thus of major economic impact. One possible route of transmission of N. caninum is through the oral uptake of sporozoite-containing oocysts which are shed in the feces of infected dogs (= definitive host). Sporozoites then enter and traverse the intestinal epithelium, infect macrophages and lymphocytes which leads to dissemination throughout the body, and transform to the rapidly proliferating and disease-causing tachyzoite stage. Subsequently, the host immune response and possibly other factors trigger stage conversion of the parasite to the slowly proliferating N. caninum bradyzoites. Bradyzoites encapsulate themselves within intracellular cysts surrounded by a solid cyst wall. Oral ingestion of tissue cysts by dogs, e.g. through infected meat, will again lead to infection and subsequent oocyst shedding. Bradyzoites can survive within a latently infected but immuno-competent intermediate host for many years without causing any clinical symptoms. However, reactivation of bradyzoites during immuno-suppression (e.g. during pregnancy) leads to severe neosporosis, namely abortion or birth of weak offspring, in cattle.

Dog-cattle- and tachyzoite-bradyzoite stages

Two different intracellular stages of *N. caninum* occur in tissues of both final (dog) and intermediate (e.g. cattle) hosts: the actively proliferating and disease-causing tachyzoite stage has been found in many different tissues and cell types, can be vertically transmitted from mother to the fetus, and accounts responsible for acute disease. The slowly proliferating and tissue cyst-forming bradyzoite stage has so far been found only in the central nervous system and muscle tissue (Fig. 1). A third stage, the sporozoite-containing oocyst, is formed within the intestinal tissue of dogs and possibly other canids, is placed into the environment by fecal shedding, and is responsible for horizontal transmission from dogs to other animals (Fig. 1). Until recently, in vitro culture of *N. caninum* was limited to the tachyzoite stage, but now tissue culture procedures have been developed to achieve tachzyoite-to-bradyzoite stage conversion, and thus tissue cyst formation, *in vitro*.

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N. caninum bradyzoites represent highly relevant targets for intervention

Bradyzoites are of particular epidemiological relevance for two reasons (reviewed in Hemphill et al., 2004; 2006): First, if the infected host becomes partially immuno-compromised, as occurs during pregnancy, bradyzoites are reactivated, which leads to bradyzoite-to-tachyzoite stage conversion, and tachyzoites will infect placental and fetal tissue, resulting in abortion or severely impaired offspring. Secondly, in dogs that consume meat infected with *N. caninum* bradyzoites, the parasite is likely to undergo sexual reproduction within the intestine. This produces oocysts that are excreted in the faeces and can contaminate soil, water and animal fodder. Thus, strategies to prevent neosporosis must also take into account the importance of bradyzoites. This has earlier led to the development of an artificially immuno-compromised mouse model for the production of *N. caninum* bradyzoites, with obvious unpleasant side effects for these animals (McGuire et al., 1997; Gondim et al., 2001). However, this laboratory animal model-based

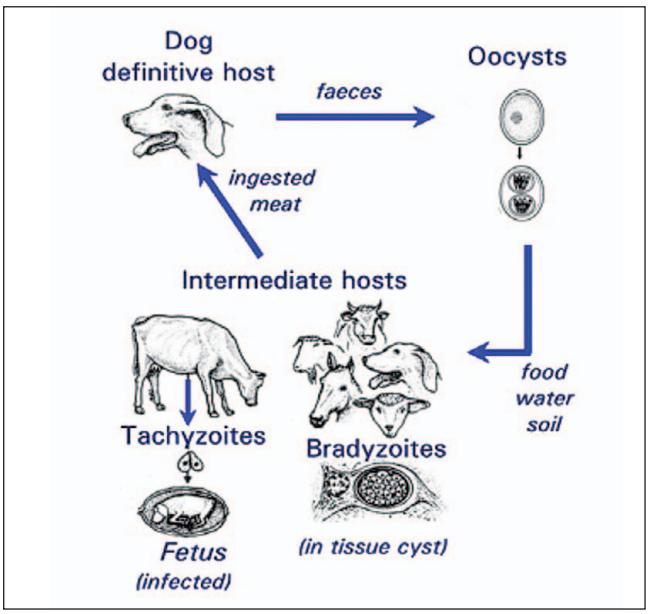

Fig. 1: Life cycle of the parasite *Neospora caninum*

method has proven rather unreliable, and requires large numbers of animals.

Nitric oxide-induced *in vitro* tachyzoite-to-bradyzoite stage conversion in cell culture

Preliminary experiments to achieve *N. caninum* stage conversion in cell culture were based on the hypothesis that the parasite would undergo tachyzoite-to-bradyzoite stage conversion under stress conditions that lead to a severe inhibition of proliferation, synthesis of tissue cyst wall components, and expression of bradyzoite-specific antigens. We systematically investigated the effects of variations in cell culture conditions for these parameters, including the use of different host cell types. Following a long phase of testing and optimizing conditions, *N. caninum* tissue cyst formation *in vitro* was first achieved by treatment of infected murine epidermal keratinocyte (MEK) monolayers with a high dose (70 µM) of the nitric oxide donor sodium nitroprusside (SNP)) for up to 8 days (Vonlaufen et al., 2002).

Subsequently, a modified and far more economical procedure was developed which enabled us to produce *N. caninum* tissue cysts containing bradyzoite stage parasites employing Vero cell cultures (Vonlaufen et al., 2004). Immunofluorescence using antibodies directed against a number of cyst wall antigens showed distinct staining of the cyst periphery, indicating that indeed a cyst wall-like structure was synthesized (Fig. 2). This was confirmed by electron microscopy, which clearly demonstrated the formation of a distinct cyst wall in bradyzoite, but not tachyzoite *in vitro* cultures (Fig. 3). Additionally, a protocol was elaborated to purify these bradyzoites from cell cultures, and this has now opened avenues to dissect this important life cycle stage of the parasite at the biochemical and molecular level without the extensive use of animal models.

Reduced infectivity of *in vitro* generated bradyzoites?

A major frustration has been the outcome of 2 separate experimental trials in which dogs were fed *in vitro* generated *N. caninum* tissue cysts obtained through mass production in Vero cells. While some of the dogs sero-converted, thus infection was initiated, we consistently failed to detect *N. caninum* oocysts in the faeces of these dogs. Either these oocysts were not produced at all, or only in low amounts and thus were not detectable using conventional coprology. These experiments actually raise concerns about the biological infectivity of these *in vitro* generated bradyzoites. However, experimental oral infection of dogs with contaminated meat does not always lead to detectable oocyst shedding (Gondim et al., 2005), thus this question remains to be clarified.

The *in vitro* approach facilitates investigations of mechanism

To our satisfaction, the application of 3R principles has come together with improved scientific impact. Other groups have taken up the methodology to generate *N. caninum* bradyzoite

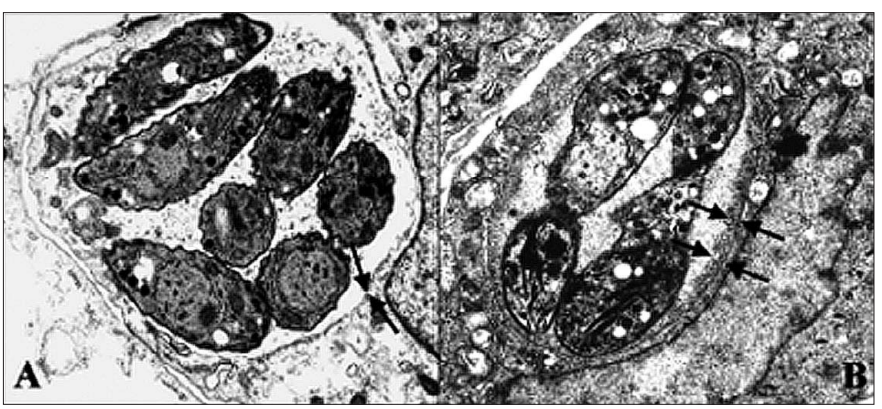

Fig. 2: *In vitro* stage conversion of *N. caninum* investigated by double-immunofluorescence.
Note the downregulation of expression of the tachyzoite antigen NcSAG1 in those parasites which exhibit expression of the bradyzoite antigen NcBAG1. Bars = 20 µm

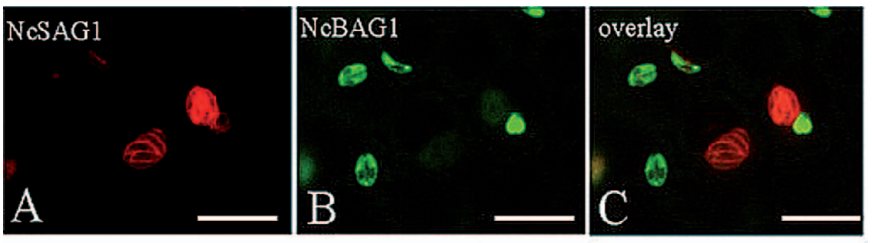

Fig. 3: Electronmicroscopical comparison of *N. caninum tachyzoites* (a) and bradyzoites (b) cultured *in vitro*. Note that both stages are located within an intracellular vacuole, but bradyzoite vacuoles (b) exhibit a defined cyst wall (thick dark structure between the arrows), which is lacking in tachyzoites (a) (cf. arrows). Bars = 1,3µm in (a) and 0.8 µm in (b).

tissue culture models by essentially the same method, in some cases, however, employing other cell types (for review see Hemphill et al., 2006). In addition, we have been able to apply this *in vitro* model to investigate differential expression of *N. caninum* antigens in several studies (Hemphill et al., 2004; 2006) and also investigated the differences in bradyzoite- and tachyzoite-host cell receptor-ligand interactions (Vonlaufen et al., 2004; 2007).

The *N. caninum* bradyzoite tissue cyst model, in conjunction with conventional tachyzoite culture, is currently successfully used:

- as a first-round *in vitro* screening tool to assess the effects and optimize the efficacy of anti-protozoal drugs such as thiazolides (Esposito et al., 2007)
- and pentamidines (unpublished)
- for the identification of potential drug targets in *N. caninum*-infected host cells
-as a model to identify stage-specifically expressed bradyzoite antigens (for review see Hemphill et al., 2006)
-for improvement of immunodiagnosis and/or vaccine development
- to investigate the stage-specific gene expression and/or localization of *N. caninum* antigens to increase our understanding of the tachyzoite-to-bradyzoite conversion process and the events leading to reactivation (Vonlaufen et al., 2004).

Outlook

As a further development of this work, we have recently established and characterized a protocol for the tissue culture of canine intestinal epithelial cells, with the aim to generate an immortalized canine intestinal cell line (3R project 85/03; Golaz et al., 2007). The overall goal of this project has been to develop a tissue culture model to produce *N. caninum* oocysts, which would enable us to generate all stages of the life cycle of this parasite *in vitro*. This would render animal models largely redundant. Work is currently in progress to achieve this goal.

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