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INVITED REVIEW

Neospora caninum and neosporosis – recent achievements in host and parasite cell biology and treatment

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Abstract

Neospora caninum is an apicomplexan parasite, which owes its importance to the fact that it represents the major infectious cause of bovine abortion worldwide. Its life cycle is comprised of three distinct stages: Tachyzoites, representing the proliferative and disease-causing stage, bradyzoites, representing a slowly replicating, tissue cyst-forming stage, and sporozoites, which represent the end product of a sexual process taking place within the intestinal tissue of the final canine host. Tachyzoites are capable of infecting a large variety of host cells *in vitro* and *in vivo*, while bradyzoites have been found mainly within the central nervous system. In order to survive, proliferate, and proceed in its life cycle, *N. caninum* has evolved some amazing features. First, the parasite profits immensely from its ability to interact with, and invade, a large number of host cell types. Secondly, *N. caninum* exploits its capability to respond to alterations in living conditions by converting into another stage (tachyzoite-to-bradyzoite or vice versa). Thirdly, this parasite has evolved mechanisms that modulate its host cells according to its own requirements, and these must, especially in the case of the bradyzoite stage, involve mechanisms that ensure long term survival of not only the parasite but also of the host cell. These three key events (host cell invasion – stage conversion – host cell modulation) represent potential targets for intervention. In order to elucidate the molecular and cellular bases of these important features of *N. caninum*, cell culture-based approaches and laboratory animal models are extensively exploited. In this review, we will summarize the present knowledge and achievements related to host cell and parasite cell biology.

Key words

Neospora caninum, host cell invasion, cell surface receptors, vaccine, host cell modulation, stage conversion, chemotherapy

Introduction

Neospora caninum (Apicomplexa, Eimeriina, Sarcocystidae) was first reported as an unidentified protozoan in dogs with encephalomyelitis and myositis (Bjerkås *et al*. 1984). The parasite was described and named by Dubey *et al*. (1988a, b), and was later also reported in various species of livestock, including cattle, sheep, goats, horses and deer (reviewed by Dubey and Lindsay 1996, Hemphill 1999). However, most importantly, the current evidence strongly indicates that infection with *N. caninum* represents a major cause of reproductive failure in cattle worldwide (Hemphill and Gottstein 2000, Dubey *et al*. 2002, Innes *et al*. 2005). The exact phylogenetical relationship of *N. caninum* to other members of the Apicomplexa has been, and still is, a matter of controversial discussions (Tenter and Johnson 1997, Dubey *et al*. 2002, Heydorn and Mehlhorn 2002).

McAllister *et al*. (1998) were the first to show that the dog is a definitive host for *N. caninum*, and this was later confirmed by Lindsay *et al*. (1999). More recently, Gondim *et al*. (2004) reported that coyotes also shed oocysts, demonstrating that other final hosts cannot be ruled out. Thus the life cycle of the parasite is not yet completely elucidated. One route of transmission in cattle is through the oral uptake of sporozoite-containing oocysts. Infection of an immunocompetent host with oocysts will not cause clinical disease, but liberated sporozoites, in analogy to other coccidian such as *Toxoplasma gondii* will most likely infect the intestinal tissue, cross the epithelium, reach blood and lymphatic vessels, and infect other cells, including macrophages and lymphocytes. In the

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initial phases of the infection, parasites will disseminate throughout the body, and in analogy to *T. gondii*, transform to the rapidly proliferating tachyzoite stage. Subsequently, the host immune response will hit the intruder, and it is believed that this is one of the factors which trigger stage conversion to the slowly proliferating *N. caninum* bradyzoites (reviewed by Buxton *et al*. 2002). Bradyzoites represent a quiescent stage of the parasite, which forms intracellular tissue cysts surrounded by a PAS-positive cyst wall. This cyst wall protects the parasite from immunological and physiological reactions on part of the host. *N. caninum* tissue cysts have been identified almost exclusively within the central nervous system, with few exceptions (Peters *et al*. 2001). Bradyzoites can survive within a latently infected, but immunocompetent, animal for many years without causing any clinical signs.

However, in special situations, such as pregnancy, bradyzoites can get reactivated, and the partial immunoincompetence of a pregnant dam, namely the decrease of an efficient Th-1 response and shift to Th-2, leads to limited suppression of the cell mediated immunity which normally keeps tachyzoite proliferation in check (reviewed by Innes *et al*. 2002, 2005; Quinn *et al*. 2002). This can contribute to the reactivation of bradyzoites and reconversion to tachyzoites, and can then lead to infection of the placenta and the foetus with *N. caninum* tachyzoites. Thus, *N. caninum* can recruit this transmission pathway by fine-tuning its differentiation state and exploiting this temporary break in host immunity to invade the foetus, usually without causing clinical disease in the dam. Transmission of *N. caninum* from mother to foetus *in utero* is highly efficient, and acute foetal neosporosis is a major cause for abortion, stillbirth, or at least clinical or subclinical disease in newborn calves (Williams *et al*. 2000, Trees *et al*. 2002). Vertical transmission can occur over successive pregnancies, and in cases where congenitally infected but clinically healthy heifers are used for breeding, parasite spreading within a herd can take place very efficiently (Björkman *et al*. 1996).

Diagnosis of *N. caninum* infection can be achieved by examination of aborted foetuses for histological lesions, and detection of parasite antigen and/or nucleotide sequences (reviewed in Hemphill 1999). Several techniques are in use for antibody detection (reviewed in Björkman and Uggla 1999). In the USA and the EU, neosporosis is reported as the leading cause of abortions in cattle (Hemphill and Gottstein 2000, Dubey *et al*. 2002). *N. caninum* infections in dairy cows may also be associated with premature culling and losses in milk production (Thurmond and Hietala 1997).

Since its discovery, the realization of the economical significance of this parasite has lead to increased efforts in elucidating how *N. caninum* interacts with its host. These interactions occur on two levels. On one hand, it is the complex relationship with the host immune system, which decides the fate of this parasite once it enters the host organism (Buxton *et al*. 2002; Innes *et al*. 2002, 2005; Quinn *et al*. 2002). On the other hand, *N. caninum* can only survive, proliferate and proceed during most stages of its life cycle as an intracellular parasite, thus the processes which lead to host cell invasion and intracellular development are of crucial importance (Hemphill 1999; Hemphill *et al*. 2004).

Neospora caninum surface components implicated in adhesion and invasion of host cells

Neospora caninum, similar to *Toxoplasma gondii*, is capable of actively invading a large variety of target cells. This process has been initially investigated *in vitro* using bovine aorta endothelial cell monolayers (Hemphill *et al*. 1996), and later for many other cell types (reviewed in Hemphill *et al*. 2004). As for *T. gondii*, the first step in the physical relationship between the parasite and the host cell is the establishment of a low-affinity contact between tachyzoite and host cell surface membrane, followed by the actual adhesion process, namely a more stable association between tachyzoites and the host cell surface (Fig. 1). Adhesion requires metabolic energy on part of the parasite, while the host cell is only passively involved (Hemphill *et al*. 1996). In order to invade their host cells, tachyzoites then reorientate themselves perpendicularly to the host cell surface membrane, and enter the host cell cytoplasm, by advancing anterior end first, until they are located in the cytoplasm, enclosed by a parasitophorous vacuole. Invasion is also an active process requiring metabolic energy solely on part of the parasite. This is highlighted by earlier findings that *N. caninum* can even infect formaldehyde-fixed host cells (Hemphill *et al*. 1996), which has also been described for *T. gondii* tachyzoites (Carruthers 2002). However, by far not all *N. caninum* tachyzoites adhering to the host cell surface will actually achieve host cell entry (Naguleswaran *et al*. 2003). Thus, specific signals and/or receptor-ligand interactions are required that enable tachyzoites to exploit their invasive capacity, and it will be a challenge in the future to elucidate those processes involved.

On the molecular level, the initial low affinity host-parasite contact is mediated, at least in part, through the two major surface antigens of *N. caninum* tachyzoites, NcSAG1 and NcSRS2 (Howe *et al*. 1998, Sonda *et al*. 1998). *In vitro* studies have shown that both polyclonal and monoclonal antibodies directed against these two surface antigens inhibit host cell adhesion and invasion (Hemphill 1996, Nishikawa *et al*. 2000). More recent studies also employing monoclonal antibodies indicate that an additional 73 kDa *Neospora* surface antigen is involved in host cell interaction (Uchida *et al*. 2004). Vaccination studies in mice showed that application of NcSRS2 and NcSAG1, either expressed in the vaccinia virus system (Nishikawa *et al*. 2001a, b) or applied as DNA vaccines (Cannas *et al*. 2003a), exhibit a protective effect against challenge infection with *N. caninum* tachyzoites. Immunisation of mice with native NcSRS2 did also protect mice from transplacental infection, as recently reported by Haldorson *et al*. (2005), confirming the potential role these surface molecules play also *in vivo*.

The surface of *N. caninum* tachyzoites exhibits considerable differences to *T. gondii* with regard to surface glycosyla-

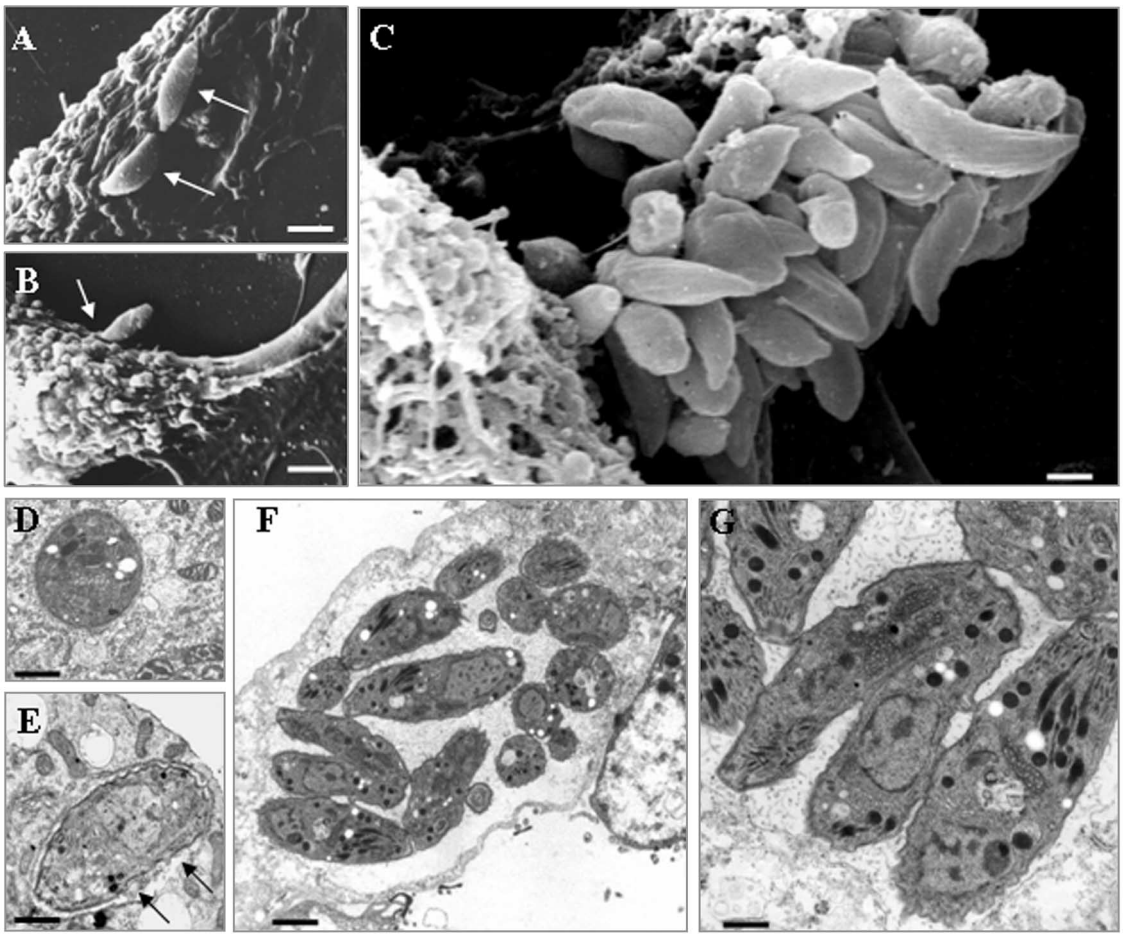

Fig. 1. Scanning (SEM) and transmission electron microscopy (TEM) of *Neospora caninum* tachyzoites invading and proliferating within mammalian cells. **A-C** show SEM micrographs: Tachyzoites first establish physical contact and then adhere to their host cells (**A**), and subsequently invade their host cells' apical end first (**B**). In **C**, tachyzoites are in the process of egress. Scale bars: A = 1.8 µm, B = 2 µm, C = 1.1 µm. **D-G** show TEM micrographs of freshly invaded parasites located within a parasitophorous vacuole surrounded by a parasitophorous vacuole membrane (**D** and **E**), larger parasitophorous vacuole containing numerous tachyzoites embedded in the tubulovesicular matrix (**F**) and larger magnification view of **F** showing a tachyzoite undergoing endodyogeny. Scale bars: D and E = 0.8 µm, F = 1 µm, G = 0.8 µm

tion. First, Baszler *et al*. (1996) have demonstrated that the surface of *N. caninum* is glycosylated, and they had shown that a monoclonal antibody directed against a periodate sensitive epitope on a 65 kDa surface protein is useful for serological diagnosis of *N. caninum* infection by competitive ELISA (Baszler *et al*. 2001). Secondly, Fuchs *et al*. (1999) have demonmonstrated the presence of surface carbohydrates on *N. caninum* tachyzoites by ruthenium red labeling, and the absence of glycans on the *T. gondii* surface. In addition, they used a panel of lectins for the identification of surface-associated glycoproteins in *N. caninum* tachyzoites, and showed that Concanavalin A (ConA) stained the surface of *N. caninum*, but not *T. gondii* tachyzoites. ConA binding sites are localized to the surface and the dense granules in *N. caninum* tachyzoites. The presence of carbohydrate modifications on antigenic proteins could potentially have implication regarding host-parasite interactions, especially with regard to masking of epitopes, and the immunological consequences thereof, and could serve as a possible explanation for the lack of extensive cross-antigenicity between the two closely related species. However, the exact functional consequences of the differential glycosylation of *Neospora* and *Toxoplasma* remain to be elucidated.

Secretory proteins/proteases and host cell invasion

Either prior, during or following host cell invasion, *N. caninum* tachyzoites, similar to *T. gondii*, sequentially discharge secretory organelles named micronemes, rhoptries and dense granules, respectively. Using *T. gondii* as a model, there have been extensive advances during the last few years on the molecular composition of these secretory organelles and how these secretion processes are accomplished (for reviews refer to Soldati *et al*. 2001, Tomley and Soldati 2001, Carruthers 2002, Mercier *et al*. 2005). In this context, proteases have

been shown to be critical for assembly and trafficking of microneme and rhoptry proteins, and parasite proteases are being considered as potential targets for chemotherapeutical intervention (Kim 2004). Invasion of host cells by *T. gondii* is through the formation of a moving junction, which selectively excludes host cell plasma membrane proteins on the basis of their membrane anchoring (Mordue *et al*. 1999), and this mechanism most likely applies to all members of the apicomplexans. In addition, it was shown that *T. gondii* tachyzoite penetration of the host cell surface membrane is dependent, and powered by, a parasite actin/myosin system, as determined by the use of specific inhibitors and parasite mutants (reviewed by Keeley and Soldati 2004). More recently it was shown that invasion itself is accompanied by proteolytic cleavage and shedding of secreted proteins as host cell invasion occurs (Binder and Kim 2004, Kim 2004, Carruthers and Blackman 2005). Among the proteases involved in these processes, subtilisin-like serine proteases have essential roles in processing of secretory components, and other studies have shown that cysteine proteases or rhomboid proteases, a newly described class of serine proteases, are important (Dowse and Soldati 2005, Dowse *et al*. 2005). It is conceivable that similar mechanisms would account for *N. caninum* invasive stages, although the respective key players have not been identified nor studied so far.

Micronemal proteins and host cell surface receptors

The first organelles to be secreted by *N. caninum* tachyzoites at the onset of adhesion are the micronemes. Microneme proteins include potentially adhesive soluble components such as NcMIC1 (Keller *et al*. 2002), NcMIC2 (Lovett *et al*. 2000), NcMIC4 (Keller *et al*. 2004), and membrane-bound microneme proteins such as NcMIC3 (Sonda *et al*. 2000, Naguleswaran *et al*. 2001). While proteolytic processing of the soluble microneme proteins has been shown to occur, there is no indication that the membrane-bound NcMIC3 is undergoing modification. Secretion of microneme contents is initiated *in vitro* by simply incubating *N. caninum* tachyzoites in medium at 37°C. This indicates that microneme secretory processes take place as soon as parasite egress from the host cells is achieved (Naguleswaran *et al*. 2001; Keller *et al*. 2002, 2004). Most likely, *Neospora* microneme proteins, in analogy to *T. gondii*, are deployed, and function as protein complexes (Carruthers 2002, Opitz and Soldati 2002, Dowse and Soldati 2005). Several *N. caninum* microneme proteins identified to date possess adhesive domains that could interact with receptors on the surface of target cells, similar to related domains found in vertebrate extracellular matrix proteins. These adhesive motifs include thrombospondin-(TSP)-like domains in NcMIC1 (Keller *et al*. 2002), integrin- and TSP-type I-like domains in NcMIC2 (Lovett *et al*. 2000), and epidermal growth factor (EGF)-like domains in NcMIC3 (Sonda *et al*. 2000).

Both, *N. caninum* and *T. gondii* have been shown to bind to their host cell surface via binding to sulfated host cell surface glycosaminoglycans (GAGs). However, despite the obvious similarities between the two species, there are distinct differences with regard to host cell interactions. First, while *N. caninum* tachyzoites preferentially bind to chondroitinsulfate GAGs, it was shown that *T. gondii* preferentially interacts with heparansulfate residues (Naguleswaran *et al*. 2002). One of the microneme proteins suggested to be mediating the contact between *N. caninum* and the host cell surface is NcMIC3. The NcMIC3 is secreted by tachyzoites at the apical tip, and remains bound to the tachyzoite surface for extended periods of time, with adhesive EGF-like domains exposed outwards (Naguleswaran *et al*. 2001). These NcMIC3-EGF-like domains, expressed in *E. coli* as a poly-His-recombinant protein, were demonstrated to bind to host cell surface chondroitinsulfates (Naguleswaran *et al*. 2002). Interestingly, vaccination of C57BL/6 mice with recombinant NcMIC3-poly-His-fusion proteins expressed and purified from *E. coli* resulted in profound protection of vaccinated animals against cerebral infection (Cannas *et al*. 2003b). However, subsequent investigations showed, that while chondroitinsulfate residues act as adhesion receptors, they do not mediate invasion of host cells (Naguleswaran *et al*. 2003).

Secondly, *N. caninum* and *T. gondii* clearly differ with regard to their susceptibility to protease inhibitors. Comparative quantification of host cell invasion events has shown that *T. gondii* invasion of host cells is impaired by the serine protease inhibitor PMSF, while inhibitors affecting other protease classes (e.g., phenanthrolin, E64, pepstatin) affect adhesion, but not invasion. In contrast, inhibition of serine-, metallo- and cysteine-proteases did not affect *N. caninum* adhesion nor invasion, but pepstatin, an inhibitor of aspartyl proteases, did have a profound impact on *N. caninum* invasion (Naguleswaran *et al*. 2003).

Thirdly, we have shown that protein-disulfide isomerase (NcPDI) is involved in *N. caninum* tachyzoite host cell interaction (Naguleswaran *et al*. 2005). The NcPDI is involved in reduction, oxidation and isomerization of intra- and intermolecular thiol-groups, and is thought to be responsible for maintaining the correct three-dimensional conformation of cysteine-rich proteins by modulating the formation of disulfide bridges. Our investigations showed that NcPDI is mainly found in the ER and in small vesiculated apical organelles resembling micronemes, but a fraction of NcPDI is also located on the surface of *N. caninum* tachyzoites. Preincubation of *N. caninum* tachyzoites with a panel of thioredoxin inhibitors, including the cell-impermeant PDI inhibitor bacitracin, has a negative impact on *N. caninum*, but not *T. gondii*, tachyzoite adhesion. The *Neospora* surface is largely composed of cysteine-rich proteins that are either constitutively or transiently expressed on the parasite surface, and the function of these proteins is highly dependent on their conformation. Therefore NcPDI could be an important factor mediating host cell interaction in *N. caninum* infection.

Stage conversion

In the immunocompetent host, *N. caninum* bradyzoites are found as a slowly proliferating and tissue cyst-forming stage, which can survive within the immunocompetent host for

many years. Bradyzoite-containing tissue cysts are orally infective, and, as immunocompetence gets impaired (such as during pregnancy), bradyzoites are reactivated, will resume proliferation and thus dissemination, and will infect the placental tissue and possibly the unborn foetus, causing abortion, stillbirth or birth of weak calves. Thus, *N. caninum* tissue cysts are in fact largely responsible for both horizontal (oral) transmission to the carnivorous final host, and vertical (transplacental) transmission to the foetus due to reactivation of quiescent bradyzoites, and are thus of prime epidemiological importance. Hence, an efficient chemotherapeutical treatment or any other means of intervention should target both tachyzoite and bradyzoite stages.

For *T. gondii*, several protocols had been earlier developed for the *in vitro* culture of the bradyzoite stage, including modulation of culture conditions by altering the pH, increasing the temperature, applying chemical stress (Soete *et al*. 1994), or mitochondrial inhibitors (Bohne *et al*. 1994). In murine macrophages, interferon-γ and sodium nitroprusside (SNP) were shown to induce stage conversion of *T. gondii* by a mechanism related to nitric oxide (NO) release (Bohne *et al*. 1994). It was also shown that increased cyclic nucleotides levels in host or parasite seem to be linked to stage conversion (Kirkman *et al*. 2001).

Neospora caninum tissue cysts have been more difficult to obtain using *in vitro* culture. Protocols developed for *T. gondii*, based on increasing the pH of the medium and treatment of infected human fibroblasts with tylosine, have yielded only few parasites undergoing stage conversion, showing that the efficiency of the differentiation process *in vitro* is rather low compared to *Toxoplasma* (Weiss *et al*. 1999). Tunev *et al*. (2002) have further improved the *in vitro* procedure by using bovine macrophages and increasing the time of culture to 9 days, thus achieving a tachyzoite-to-bradyzoite conversion rate of 14% based on the immunofluorescent detection of BAG1 expression. Using NcSAG1/BAG1 double immunofluorescence labeling they also provided evidence that vacuoles containing BAG1-positive bradyzoites may develop only if the host cell is invaded by a parasite that has already began tachyzoite-to-bradyzoite stage conversion prior to host cell entry, and that cyst formation does not appear to occur through transformation of parasitophorous vacuoles that originally contain tachyzoites (Tunev *et al*. 2002).

Vonlaufen *et al*. (2002) has established an alternative model, which is based on the use of *N. caninum* Liverpool isolate and SNP-treated murine epidermal keratinocytes as host cells. Infected keratinocytes are treated with SNP for up to 8 days. The SNP releases NO that reacts with the iron sulfur centres of several proteins involved in electron transport of the respiratory chain and heme iron of cytochrome C oxidase. This results in a decrease of ATP formation and in a diminished binding of oxygen to cytochrome C oxidase (Cooper 1999). The adaptation of the parasites to a decreased energy production and to an anaerobic environment may trigger the differentiation process, and thus results in the formation of slowly dividing bradyzoites, which consume less energy. It was demonstrated for *T. gondii* that stage conversion also occurred in host cells lacking mitochondrial functions, indicating that it is the parasite mitochondrium which mediates this effect (Bohne *et al*. 1994), and most likely the same is true for *N. caninum* in this culture system. *N. caninum* tachyzoite-to-bradyzoite *in vitro* cultivation has been adapted for the use of Vero host cells, a culture system that allows the separation of bradyzoites from host cells (Vonlaufen *et al*. 2004). This procedure allowed for the isolation of bradyzoites, and host cell invasion assays demonstrated significant differences between tachyzoites and bradyzoites with regard to invasive capacity and requirements of host cell surface receptor molecules, e.g. sialic acid residues. In addition, we demonstrated that a number of dense granule proteins are secreted during *in vitro* stage conversion and are incorporated into the cyst wall (Vonlaufen *et al*. 2004). A similar procedure for obtaining bradyzoites in an efficient way in cell culture was established by Risco-Castillo *et al*. (2004), by using MARC145 monkey kidney epithelial cells as host cells. The same group recently reported on the identification and cloning of the NcSAG4 gene, an orthologue to the *T. gondii* TgSAG4 gene, which is the first reported gene to be expressed specifically during the *N. caninum* bradyzoite stage (Fernández-García *et al*. 2006).

Host cell modulation and parasite-host cell crosstalk

Once inside the host cell, *N. caninum* resides within a parasitophorous vacuole (PV), surrounded by a parasitophorous vacuole membrane (PVM), which is essentially derived from the host cell surface membrane. There are many similarities with *T. gondii* infected cells, where the PV resists acidification and phagolysosomal maturation (Mordue and Sibley 1997, Mordue *et al*. 1999). *T. gondii* and *N. caninum* containing PVs shuttle from the host cell membrane to a location in the near vicinity of the host cell nucleus, and they become physically associated with mitochondria and the ER (Hemphill *et al*. 1996, Sinai *et al*. 1997). Rhoptry proteins, discharged at the early phase of vacuole formation, are probably involved in the biogenesis of the PV. Of these, *T. gondii* ROP2 has been reported to insert into the vacuole membrane and mediate anchoring to host cell mitochondria (Sinai and Joiner 2001). Following invasion, the lumen of the vacuoles of both parasites as well as its membrane is extensively modified through secretory products, most likely originating from rhoptries and dense granules (Cesbron-Delauw 1994, Hemphill *et al*. 1998, Vonlaufen *et al*. 2004). Since in *T. gondii*-infected cells, the PVM actively recruits host cell mitochondria and ER, protocols have been developed for the enrichment of this organellar membrane complex (Sinai *et al*. 1997).

In *N. caninum* infected Vero cells, host cell mitochondria are also occasionally found in the vicinity of the PVM, but a close physical interaction between the PVM and host cell mitochondria like in *T. gondii* infected fibroblasts has not been observed, neither in bovine endothelial cells infected with *N. caninum* tachyzoites (Hemphill *et al*. 1996), nor in Vero cells infected with *in vitro* cultured bradyzoites (Hemphill *et*

al. 2004, Vonlaufen *et al*. 2004). This was also found earlier in a comparative ultrastructural study by Speer *et al*. (1999), which reported that in cells infected with *T. gondii* VEG strain tachyzoites, several host cell mitochondria were closely associated with the PVM, whereas only few host cell mitochondria were found in the vicinity to the PVM surrounding *N. caninum* tachyzoites. In addition, secreted NcGRA7 was found to be associated with the PVM (Hemphill *et al*. 1998).

There is accumulating evidence, that the PVM, its constituents, as well as secretory parasite molecules passing through this membrane into the host cell cytoplasm, are involved in crosstalk and manipulation of host cell functions. For instance, in *T. gondii* infected macrophages, LPS-induced cytokine signaling is blocked by the presence of tachyzoites (Denkers *et al*. 2004). It has been shown that external addition of parasite lysates and secretion products do not mediate this suppression. Furthermore, killing of intracellular parasites by drug treatment relieved the blockage of LPS-induced secretion of TNF-alpha (Butcher and Denkers 2002). Luder *et al*. (2001) showed that *T. gondii* down-regulates MHC class II gene expression and antigen presentation by murine macrophages via interference with nuclear translocation of STAT1alpha. The same group has also demonstrated that *T. gondii* infection of macrophages results in reduced expression of inducible nitric oxide synthase and facilitates replication in activated macrophages (Luder *et al*. 2003a), and that infection in neural antigen-presenting cells inhibits MHC class II expression by down-regulating the class II transactivator CIITA (Luder *et al*. 2003b). Other investigators found that *T. gondii* inhibits host cell apoptosis by inducing the activation of the transcription factor NFkappaB, which in turn is regulating the expression of inhibitors of apoptosis in the host cell (reviewed by Sinai *et al*. 2004). The activation of NFkappaB pathway by *T. gondii* correlates with the localization of phosphorylated I kappaBalpha at the PVM (Molestina *et al*. 2003), and more recently, Molestina and Sinai (2005) detected a novel kinase activity at the *T. gondii* PVM capable of phosphorylating host I kappaBalpha. In this context, Goebel *et al*. (2001) reported that inhibition of host cell apoptosis by *T. gondii* is accompanied by reduced activation of the caspase cascade and alteration of poly(ADP-ribose) polymerase expression. However, the outcome of host cell modulation by *T. gondii* can differ, dependent on the virulence of the infecting strain. Hisaeda *et al*. (1996, 1997) showed that the less virulent Beverly strain inhibited apoptosis in infected macrophages, while the more virulent RH strain induced apoptosis. The authors suggested that expression of HSP65 in host cells was responsible for this difference. However, the timing of HSP expression appears to be critical. Nevertheless, expression of HSP65 seems to be another common mechanism for preventing apoptosis during infection of mammalian cells with parasites (reviewed by Heussler *et al*. 2001).

Altogether, these findings indicate that messages to terminate host signaling may be delivered internally, possibly across the PVM. How this is achieved is not clear. There is only little information regarding the composition of the PVM proteome, and information on proteins that are secreted into the host cell cytoplasm across the PVM is sparse. In *T. gondii*, ROP2 and GRA5 are known to span the PVM, partially penetrating into the host cell cytosol (Beckers *et al*. 1994, Lecordier *et al*. 1999). Such PVM-spanning molecules could assist in modulating host cell signaling pathways. In addition, it has been hypothesized, that parasite molecules are specifically directed across the PVM into the cytoplasm, where they could target host signaling cascades (Denkers *et al*. 2004).

Host cell modulation by *N. caninum* has not yet been investigated extensively. However, distinct differences to *T. gondii* have been noted. It was demonstrated that infection of IFN-γ-treated BALB/3T3 clone A31 fibroblasts with *N. caninum* tachyzoites *in vitro* causes apoptosis (Nishikawa *et al*. 2001c). Apoptosis of *N. caninum* infected and IFN-γ-treated cells was shown to be associated with increased DNA-fragmentation, and increased caspase-3 and caspase-8 activity, and the administration of respective inhibitors inhibited cell death. The reduction in cell viability was prevented with the addition of anti-mouse FasL monoclonal antibody (Nishikawa *et al*. 2002). However, this aspect merits closer investigations. For sure, inhibition of apoptosis is a critical issue for the chronic phase of infection, during which *N. caninum* bradyzoites proliferate only slowly within a host cell, and form tissue cysts that can persist within infected tissue for years. These *N. caninum* bradyzoites, as well as their host cells, are predestined for long term survival. It is conceivable, that host cells are modulated accordingly, by either utilizing the PVM as a signaling platform, or by actively secreting bioactive parasite derived factors into the cytoplasm.

Experimental parasite cell affection by chemotherapy

The need for the development of effective pro- or metaphylactive measures against bovine neosporosis has been widely addressed and discussed (Liddell *et al*. 1999, Gottstein *et al*. 2001, Greif *et al*. 2001, Innes *et al*. 2002, Kritzner *et al*. 2002). So far, a relatively wide range of chemotherapeutically active components has been tested against *N. caninum*. Thus, Lindsay *et al*. (1994) have examined potential activities of 43 chemotherapeutic agents against *in vitro* cultivated *N. caninum* tachyzoites, including sulfonamides, dihydrofolate reductase/thymidylate synthase inhibitors and ionophorous, macrolide and tetracycline antibiotics. The highest activity was exhibited by clindamycin. Some compounds are known to kill the parasite on cell culture-based assays (Lindsay *et al*. 1994, Dubey 2003). The crucial issue so far has been that most of these experiments relied upon *in vitro* cultivated *N. caninum* tachyzoites as target organisms, which, by definition, are much easier accessible for chemical affection than bradyzoites. Also *in vivo* experiments in the mouse model have primarily tackled the tachyzoite-dependent phases of infection and disease. The aim of such treatment was thus to primarily demonstrate the prevention of parasite (tachyzoite) dissemination – especially in its cerebral form – rather than prevention of later stage parasite establishment such as formation of

tissue cysts. The strategic interruption of parasite dissemination, e.g. during pregnancy in cattle or dogs, may putatively prevent the diaplacental passage of the parasite or may considerably reduce the abortion risk during conatal neosporosis. Explorative investigations had shown that toltrazuril, a triazinone derivative, can be effective against experimental neosporosis in the murine (Eperon *et al*. 1999, Gottstein *et al*. 2001) and bovine (Kritzner *et al*. 2002) model, and that an efficient metaphylaxis requires at least a T-cell-mediated immunological support (Ammann *et al*. 2004). This may be explained by a parasitostatic rather than a parasitocidal effect of the compound. Toltrazuril is known to inhibit the transfer of electrons along the mitochondrial respiratory chain. However, this is unlikely to be its only specific mode of action (Harder and Haberkorn 1989). Various types of immunological deficiencies had been addressed to investigate the importance of specific components of the immune system to control the infection. Interferon-γ knockout mice developed acute and lethal neosporosis within two weeks, and interleukin-12 knockout mice had exhibited a high susceptibility to acute neosporosis (Khan *et al*. 1997, Baszler *et al*. 1999, Nishikawa *et al*. 2001d, Ritter *et al*. 2002). Nude mice are very susceptible to *N. caninum* and develop acute neosporosis (Yamage *et al*. 1996, Shibahara *et al*. 1999). B-cell deficient C57BL/6-μMT mice have been proven to be highly susceptible to neosporosis as well (Eperon *et al*. 1999). Toltrazuril was suggested to act at least against tachyzoites, and appeared not to abrogate the development of immunity (Greif 2000). Therapy thus did not affect the parasite before immunocompetent mice mounted an antibody response. This, however, was significantly weaker in treated than in untreated animals. Toltrazuril appears not to exhibit an immediate knockdown effect, thus in order to become active against the parasite it requires the immune system (Ammann *et al*. 2004). Nevertheless, the tachyzoites were probably eliminated before having invaded the brain. In untreated animals, the infection was also clinically contained, although brain damage was not completely prevented and a few single tachyzoites were visible by immunofluorescence in one untreated mouse. Conclusively, the results obtained with athymic nu¯/nu¯ mice demonstrate that toltrazuril could not clear the infection, but resulted in a marked delay of parasite dissemination and disease in the T-cell deficient host (Ammann *et al*. 2004). T-cells thus seemed to play a crucial role in the protection against *N. caninum* (Baszler *et al*. 1999, Nishikawa *et al*. 2001d).

The role of immunity to control, or to permit, parasite recrudescence during pregnancy may be crucial for the occurrence of foetal infection especially in bovines. Thus the outcome of neosporosis during bovine gestation – with an altered immune response – appeared dependent on the time of infection, among other features (Innes *et al*. 2000). In mice, the Th2 cytokine bias observed in pregnant animals (Athanassakis and Iconomidou 1996) may favour the activation capacity of *N. caninum*. Thus, Long and Baszler (2000) demonstrated in *N. caninum* infected mice that IL-4 neutralization before pregnancy – concomitant with the inoculation of an avirulent strain of *N. caninum* – decreased congenital transmission after a challenge during pregnancy. Rettigner *et al*. (2004) studied the immune response in mice chronically infected with *N. caninum* during successive pregnancies and in mice acutely infected during an ongoing pregnancy. Vertical transmission was demonstrated in chronically infected mice after the first pregnancy but the rate of foetal infection fell after further pregnancies.

We now recently showed that toltrazuril can contribute to the control of congenital neosporosis in mouse dams (Gottstein *et al*. 2005). As a first finding, toltrazuril-treatment significantly reduced pre- and perinatal losses due to experimental *N. caninum* infection when compared to nontreated dams in their first gestation. Furthermore, the mothers themselves appeared to be protected from potential clinical consequences of the infection, as all dams remained clinically healthy throughout the experimental period, while a few dams died of the infection in the nontreated group. Therefore, the question arises as to whether pregnant mice are basically more susceptible to disease than nonpregnant animals. In previous experiments, symptomatic disease in immunocompetent, nonpregnant mice almost exclusively resulted from much higher infection doses as used in the present experiments (Gottstein *et al*. 2001), whereas reduced immunocompetence markedly increased susceptibility to disease (Yamage *et al*. 1996, Eperon *et al*. 1999, Ammann *et al*. 2004). Conversely, toltrazuril-treatment had no effect on a repeated later pregnancy in mice subjected to the same initial infection and treatment protocol. As discussed by Rettigner *et al*. (2004), vertical transmission efficiency is markedly reduced under a chronic infection status and increasing number of pregnancies. We obtained similar results, as offsprings born from even nontreated (but chronically infected) mice had no detectable parasite DNA in their brain, although these offspring animals had become seropositive. However, seropositivity may have been acquired diaplacentally through antibody transfer by seropositive dams during foetal development. This was also in agreement with the findings of Cole *et al*. (1995) who observed, in mice inoculated during their first pregnancy, a transmission rate reduction of 25% after a second gestation and an absence of transfer after a third or a fourth gestation.

Besides reducing abortion and infertility rates in pregnant mice, toltrazuril-treatment also affected directly the infection course in the foetuses, best demonstrated by the lack of postnatal death in offspring of toltrazuril-treated mothers versus postnatal death of some of the newborns of nontreated mothers (Gottstein *et al*. 2005). Although infected dams may remain carriers of the parasite, they seem not to act as sources of infection for the foetus in later pregnancies, conversely to the situation found in naturally infected cattle (Gottstein *et al*. 2005).

Conclusions/outlook

Once inside its host cell, *N. caninum* will either undergo proliferation (tachyzoites), or tissue cyst formation (bradyzoites), and both events are dependent on a host cell, which does exactly what the parasite wants it to do. Elucidating the mech-

anisms leading to host cell invasion and host cell manipulation is crucially important for the development of possible means of intervention, either by prevention of disease (e.g., by vaccination) or by treatment (e.g., chemotherapy). Studies on *N. caninum* and its interactions with the host have been immensely accelerated by similar investigations on *T. gondii*, which serves as the number one apicomplexan model organism and currently represents one of the best characterized protozoan parasites. For *N. caninum*, cell culture based approaches have provided, and will continue to provide, important information on the cellular and molecular mechanisms defining the host-parasite relationship, and have already provided clues on potential targets for prevention and intervention. However, the true significance of these targets can only be conclusively assessed in a valuable animal model. In terms of strategic control options, toltrazuril-treatment, e.g., of an acute *N. caninum* infection during pregnancy in mice leads to a clear reduction of foetal losses and a marked reduction of transplacental transmission of the parasite to the foetus or foetal brain, respectively. An explorative study to assess the efficacy of toltrazuril sulfone (ponazuril) has been carried out in calves experimentally infected with *N. caninum* (Kritzner *et al*. 2002) and had proven some efficacy. The final question to be respectively addressed is if newborn calves are parasitised with tachyzoite stages predominantly, which would render the chemotherapeutical targeting much easier. Conversely, tissue cysts and bradyzoites would hardly be affected by conventional drugs, and treatment thus would not be able to create parasite-free offsprings.

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