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Proteins mediating the *Neospora caninum*-host cell interaction as targets for vaccination

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1. ABSTRACT

Neospora caninum is an apicomplexan parasite that is capable of infecting, a wide range of tissues. The fact that *Neospora* represents an important abortion-causing parasite in cattle has transformed neosporosis research from an earlier, rather esoteric field, to a significant research topic, and considerable investments have been made in the last years to develop an efficacious vaccine or other means of intervention that would prevent infection and abortion due to *N. caninum* infection in cattle. Antigenic molecules associated with proteins involved in adhesion/invasion or other parasite-host-cell interaction processes can confer protection against *Neospora caninum* infection, and such proteins represent valuable targets for the development of a vaccine to limit economical losses due to neosporosis. Although not ideal, small laboratory animal models that mimic cerebral infection, acute disease and fetal loss upon infection during pregnancy have been used for the assessment of vaccine candidates, in parallel with studies on experimental infections in cattle. Herein, we review and critically assess these vaccination approaches and discuss potential options for improvements.

2. *NEOSPORA CANINUM* AND NEOSPOROSIS

Neospora caninum (Apicomplexa: Eimeriina: Sarcocystidae) was first reported as an unidentified protozoan in dogs with encephalomyelitis and myositis (1). The parasite was described and named by Dubey et al. (2, 3), and was later also reported in various species of livestock, including cattle, sheep, goats, horses and deer (reviewed in 4-7). In the USA and Europe, neosporosis is reported as the leading cause of abortions in cattle (5, 7), thus the disease represents an important veterinary health problem and is of high economical significance.

Dogs and coyotes have been identified as definitive hosts for *N. caninum* (8-10). One possible route of transmission in cattle is through the oral uptake of sporozoite-containing oocysts. While not causing clinical disease in an immuno-competent animals, liberated sporozoites are believed to infect the intestinal tissue, cross the epithelium, reach blood and lymphatic vessels, and infect other cells, including macrophages and lymphocytes. While this has never been shown conclusively, a recent in vitro study on cultured canine intestinal epithelial cells has

demonstrated that *N. caninum* tachyzoites and bradyzoites are capable of infecting, and proliferating within, canine intestinal epithelial cells in vitro (11). Following dissemination, sporozoites transform to the rapidly proliferating tachyzoite stage. Subsequently, the host immune response will hit the intruder, and this could be one of the factors that trigger stage conversion to the slowly proliferating bradyzoites (12). Bradyzoites represent a quiescent stage of the parasite, forming intracellular tissue cysts surrounded by a PAS-positive cyst wall, almost exclusively within the central nervous system, and possibly in muscle tissue (13). Bradyzoites can survive within a latently infected but immuno-competent animal for many years without causing any clinical signs. However, in special situations, such as pregnancy, bradyzoites can get reactivated, and the loss of an efficient Th-1 response of a pregnant dam possibly leads to limited suppression of the cell mediated immunity which normally keeps tachyzoite proliferation in check (14), possibly contributing to the reactivation of bradyzoites and transplacental infection of the fetus. 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 fetus, usually without causing clinical disease in the dam. *In utero*-transmission of *N. caninum* is highly efficient, rendering neosporosis an important veterinary medical problem.

The economic impact of neosporosis upon the cattle industry is rather extensive (reviewed in 7). It has been estimated that in California approximately 40,000 abortions could be due to neosporosis, providing an estimated loss of 35 million dollars per year. Beside the loss caused by the abortion itself, reduced milk yield, premature culling and reduced post-weaning weight gain in beef calves have to be considered. In Australia and New Zealand, losses are thought to be more than $100 million Australian Dollars per year and in the Netherlands estimates of 19 million Euros have been reported. A recent study estimating the median annual losses due to *N. caninum* in the Swiss dairy cow population led to the result of 9.7 million Euros (15).

Thus, the economic importance of neosporosis, especially in cattle, has lead to research on the development of strategies for prevention and treatment of *N. caninum* infection. Vaccination and chemotherapy have been identified as economically promising options (15, 16). *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, and they represent potential targets for vaccination and/or chemotherapeutic intervention (17).

3. HOST CELL ADHESION/INVASION BY *N. CANINUM* AND RELATED APICOMPLEXAN PARASITES

Neospora caninum, similar to the closely related *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 (18), and later for many other cell types (7, 18, 19). The basic mechanisms on how host cell invasion is achieved are clearly conserved among the phylum Apicomplexa, and *T. gondii* has been the prime model apicomplexan to investigate this process at the molecular level (20). Host cell invasion is composed of defined steps outlined below. Although subtle, distinct variations have been observed between *T. gondii* and *N. caninum*, these can account for the differences in the biological characteristics of the two species (17, 21). Nevertheless, the mechanisms that govern the physical interaction between parasite and host cell represent potential targets for vaccination and/or treatment strategies in both toxoplasmosis and neosporosis. The major key players in the interaction between parasite and host cell are represented by components of a set of secretory organelles, which are named micronemes, rhoptries and dense granules (see Figure 1A, B).

3.1. Initial adhesion is mediated by surface antigens (SAGs)

As for *T. gondii*, the first step in *N. caninum* host cell invasion is the establishment of a low-affinity contact between tachyzoite and host cell surface membrane (18). This contact is mediated, at least in part, through the two major surface antigens NcSAG1 and NcSRS2, as shown by in vitro studies employing respective monoclonal and polyclonal antibodies (22, 23). This initial SAG-mediated adhesion (see Figure 1 C, D) is reversible, permitting the parasite to disengage, if the particular cell type encountered is suboptimal, or the conditions for invasion are otherwise not ideal (20).

3.2. Apical attachment is mediated by microneme proteins (MICs)

In order to establish a firmer binding to their host cells, tachyzoites re-orientate themselves perpendicularly to the host cell surface membrane (Figure 1 E). Treatment of *T. gondii* tachyzoites with cytochalasin D, a compound that arrests gliding motility by acting on actin filaments (and thus acts on the actin-myosin machinery necesserary for motility), resulted in apically attached parasites, which were unable to invade their host cell. Apical host cell contact is mediated by proteins secreted from micronemes, small cigar-shaped organelles located at the anterior pole of all apicomplexans (Figure 1 B, F). Many micronemal MIC-proteins exhibit conserved adhesive domains, such as thrombospondin (TSP)-like, epidermal growth factor (EGF)-like, integrin-like (I)-like, and lectin-like domains, which are known to mediate protein-protein or protein-carbohydrate interactions in other systems. In fact, these domains interact with receptors on the surface of target cells, similar to related domains found in vertebrate extracellular matrix proteins (24). More recent studies demonstrated structural aspects of these host cell interactions and binding of microneme proteins to sialic acid residues, or formation of microneme complexes via EGF domains, at the atomic level, and this information is to be exploited for the development of potential therapies (25-27). MICs exist in either membrane-bound or soluble forms, and several MICs have been demonstrated to build up complexes, which are formed in the endoplasmatic reticulum, then travel through the secretory pathway, and

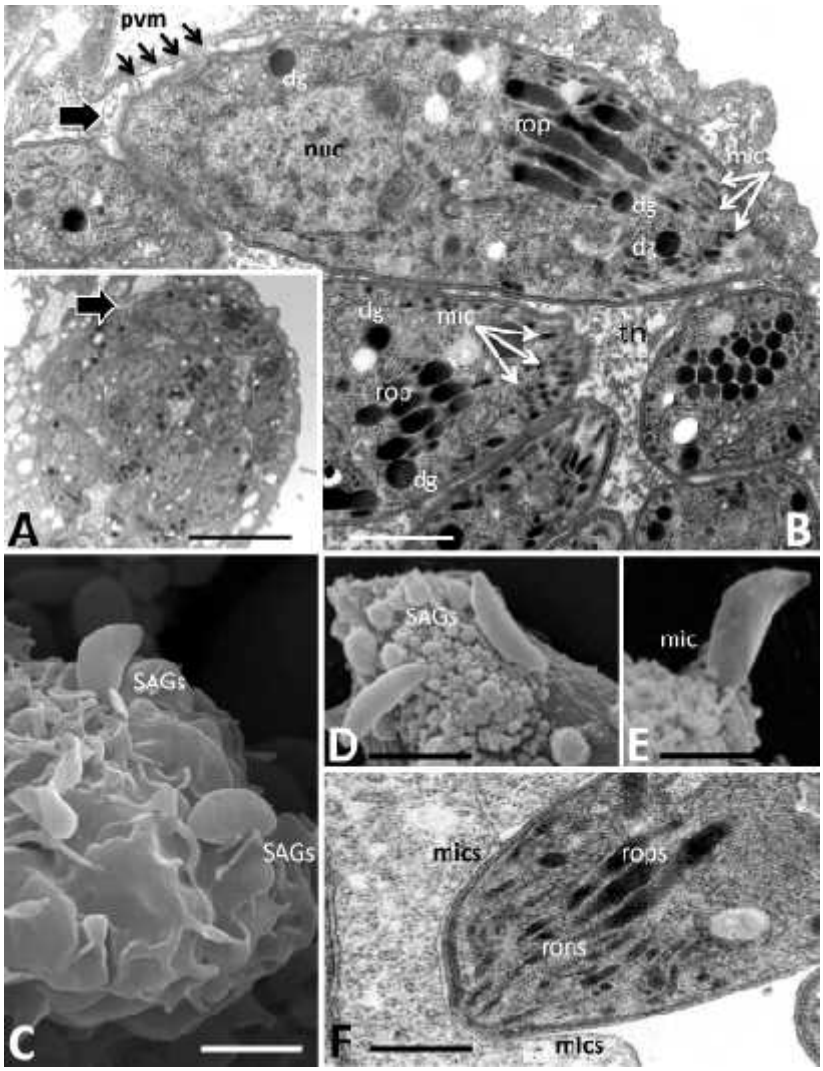

Figure 1. SEM and TEM of initial phases of host cell invasion by *Neospora caninum* tachyzoites. A and B shows a low magnification view of a large parasitophorous vacuole of tachyzoites in Vero cells (A) and a higher magnification view of the same vacuole (B). Note the three distinct secretory organelles that play a major role in host cell interaction, namely the rod-shaped rhoptries (rop), the small cigar-formed micronemes (mic), both at the anterior end of the parasites, and the dense granules (dg), located at both the anterior and posterior end. Nuc = nucleus, the bold arrow in (A) and (B) points to the corresponding location in the micrographs, small arrows point to the parasitophorous vacuole membrane (pvm), tn = tubular network forming the vacuole matrix. C and D show SEM images of liberated tachyzoites that adhere to the cell surface of cultured Vero cells, and interaction that is mediated via their major parasite surface antigens (SAGs). 1E shows a tachyzoite oriented perpendicularly to the host cell surface through the interaction of microneme proteins (mics) that serve as host cell surface-binding factors and which are secreted anteriorly at the onset of host cell invasion. F shows a TEM image of the anterior end of a tachyzoite at the initiation of host cell invasion, mediated by the sequential secretion of micronemal (mics) and rhoptry proteins (rops). Bars in A=12µm, B= 0.6µm, C=10µm, D=12µm, E=10µm, F=0.6µm.

are finally released prior to invasion. In *T, gondii*, four complexes have been identified so far. Their specific functions have been elucidated by generating conditional knockouts for the genes coding for the components of these complexes.

Most likely, *Neospora* microneme proteins, in analogy to *T. gondii* and other apicomplexans are also deployed and function as protein complexes (28). *N. caninum* apical secretion of microneme contents in vitro is initiated by simply incubating tachyzoites in medium at 37ºC. This indicates that microneme secretory processes take place as soon as parasite egress from the host cells is achieved (29, 30), and also explains why *N. caninum* looses infectivity rapidly upon extracellular maintenance (17, 31).

Molecular studies have suggested early on that MICs could represent interesting vaccine candidates. *T. gondii* mutants with genetically ablated TgMIC2 and TgMIC2-associated protein (M2AP) exhibited substantially

reduced invasive capacities (32, 33). Cerede et al. (34) showed that single deletion of the TgMIC1 gene decreased invasion in fibroblasts in vitro, whereas TgMIC3 deletion had no effect. Individual disruption of TgMIC1 or TgMIC3 genes slightly reduced virulence in mice, whereas doubly depleted parasites were severely impaired in virulence and conferred protection against subsequent challenge. Single substitution of two critical amino acids in the chitin binding-like (CBL) domain of TgMIC3 abolished TgMIC3 binding to cells and generated the attenuated virulence phenotype. These findings identified the CBL domain of TgMIC3 as a key player in toxoplasmosis. However, the *N. caninum* MIC3 homologue does not possess a conserved CBL domain (35), and adhesion to host cells is mediated by 4 consecutive EGF-like domains (36). Nevertheless, the synergistic role of microneme proteins in virulence probably accounts for all apicomplexans, including *Neospora,* and supports the idea that parasites have evolved multiple ligand-receptor interactions to ensure invasion of different cell types during the course of infection (28). Several *N. caninum* MICs identified to date possess these adhesive domains that are also found on *T. gondii* homologues (29, 35, 37, 38). However, *N. caninum* and *T. gondii* exhibit a different preference for host cell surface glycosaminoglycans (GAGs), as *N. caninum* tachyzoites preferentially interact with chondroitinsulfate, and NcMIC3 does the same via its EGF-like domains. In contrast, *T. gondii* tachyzoites bind preferentially to heparansulfate residues (39). Sialic acid has been identified as a key recognition factor for *T. gondii,* and Friedrich et al (25) recently expanded this observation by reporting on the role of a novel protein family containing microneme adhesive repeat (MAR) domains that act as sialic acid-binding lectins during host cell invasion in apicomplexans.

Several vaccination studies documented the importance of microneme proteins as vaccine candidates against *T. gondii* infection. In one of them, Ismael et al (40) showed that intramuscular DNA vaccination of mice with TgMIC3 elicited a strong specific immune response and provided effective protection against *T. gondii* infection. In a subsequent paper, Ismael et al (41) demonstrated that the *T. gondii* MIC1-3 double knock-out mutant was a powerful vaccine and protected mice against chronic and congenital toxoplasmosis. Recently, vaccination of mice with the *T. gondii* MIC1-3 double knock out mutant was also shown to protect mice against lethal *N. caninum* challenge infection (42).

3.3. Host cell entry is achieved by formation of a moving junction composed of microneme and rhoptry components

In *T.gondii,* another MIC protein, apical membrane antigen 1 (TgAMA1), forms a complex with three rhoptry neck proteins (TgRONs). This complex then associates to the moving junction, a structure between parasite and host cell surface membrane, which is reminiscent of a thight junction in mammalian cells, and which slides over the parasite as it invades (43). When TgAMA1 expression is down regulated to 1% of original levels, apical attachment is not affected, but the moving junction is not formed, resulting in inhibition of host cell invasion (44). The moving junction selectively excludes host cell plasma membrane proteins on the basis of their membrane anchoring (45). Moving junction formation as a tool to invade the host cell is most likely very conserved among apicomplexans: *Plasmodium falciparum* AMA1 was also found to associate with a RON4-homologue to form a moving junction (46). It is likely that similar mechanisms would account for *N. caninum* tachyzoites, as they move into the host cell cytoplasm by pulling themselves along the host cell surface membrane, and thereby also leaving shedded material containing surface antigens such as e.g. SAG1 (Fig. 2A). The NcAMA1 homologue has been identified by Zhang et al (47), and antibodies against NcAMA1 inhibit host cell invasion by *N. caninum* tachyzoites and *T. gondii* tachyzoites in vitro, implicating its potential usefulness as a vaccine candidate.

3.4. Formation of the parasitophorous vacuole membrane (PVM) is governed by rhoptry (ROPs) and dense granule (DG) proteins

Simultaneously, or right after the formation of the moving junction, other rhoptry proteins (ROPs) are injected into the host cell cytoplasm at the invasion site. Some ROPs remain associated with small vesicles that fuse with the developing parasitophorous vacuole membrane (PVM), whereas others remain soluble and are targeted to other sites within the host cell (20). The parasite actively penetrates by pulling transmembrane MIC-complexes and/or the moving junction towards its posterior end, and thereby invaginates the host cell surface membrane to create a vacuole (Figure 2A). During the invasion process, rhomboid proteases are responsible for sheding of the microneme proteins from the posterior end. Proteases involved in this processing constitute interesting targets for chemotherapeutic intervention (20, 48). *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 such as cytochalasin D and parasite- and host cell mutants (20, 49, 50). Cytochalasin D also inhibts host cell invasion by *N. caninum* tachyzoites (17). However, under normal circumstances, by far not all *N. caninum* tachyzoites adhering to the host cell surface will actually achieve entry, and the invasion process is less efficient compared to *T. gondii* (31). Thus, specific signals and/or receptor-ligand interactions are required to enable tachyzoites to enhance their invasive capacities. These receptors or ligands represent suitable targets for the development of preventive/interventional measures by vaccination and/or chemotherapy.

3.5. Intracellular parasitism and parasite-host cell communication.

Once inside the host cell, *N. caninum* resides within a parasitophorous vacuole (PV), surrounded by the PV-membrane (PVM; see Figure 2B-D). There are many similarities with *T. gondii* infected cells, where the PV resists acidification and phagolysosomal maturation (45, 48). Following invasion, the lumen of the vacuole as well as its membrane are extensively modified through secretory products, most likely originating from rhoptries and dense granules (51, 52). *T. gondii* containing PVs shuttle from the host cell membrane to a location in the near vicinity of the

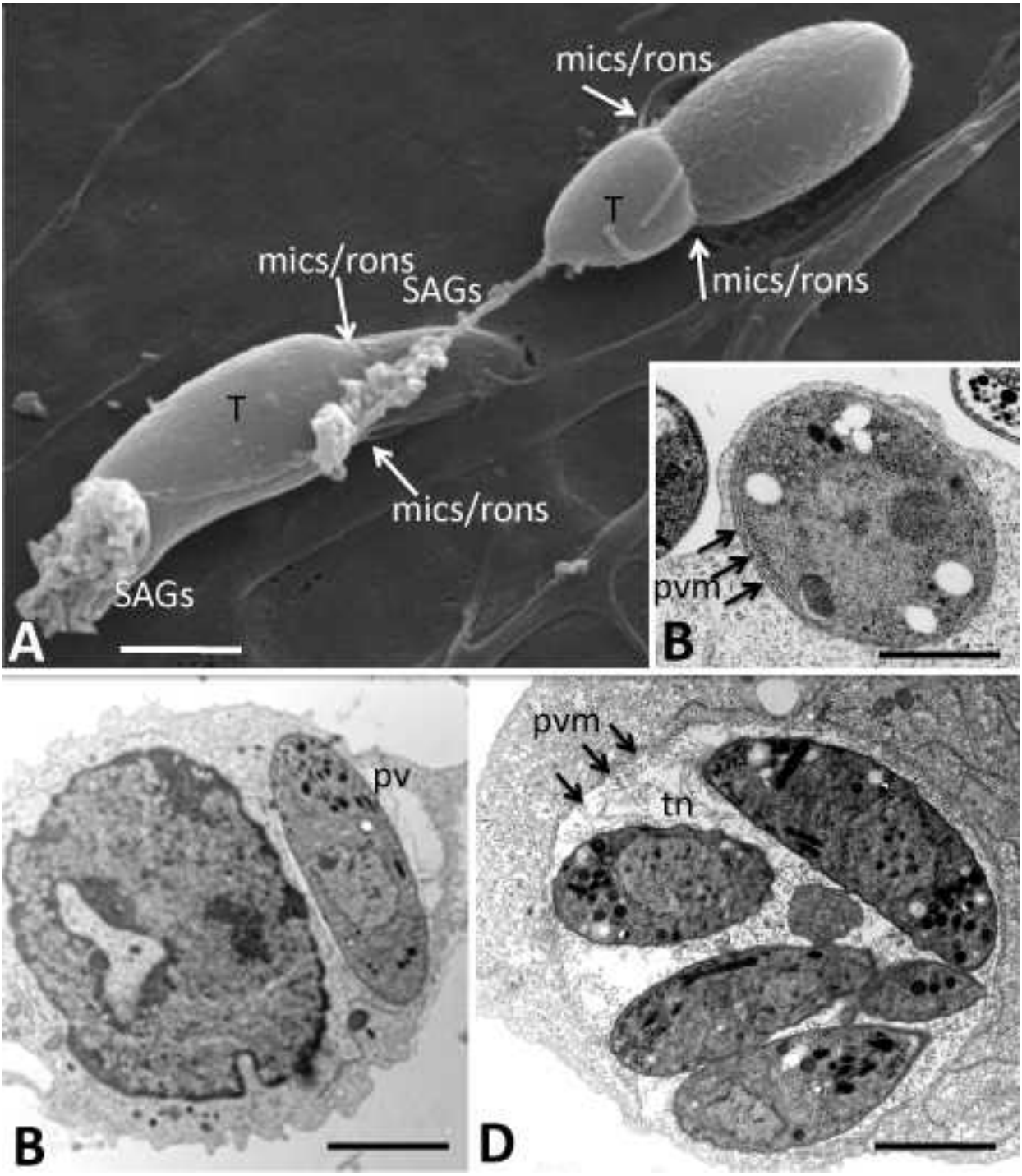

Figure 2. SEM and TEM of the host cell invasion process. A shows two *N. caninum* tachyzoites (T) invading a human fibroblast cell in vitro, moving into the host cell cytoplasm from left to right. Note the presence of a moving junction (marked by arrows), that is, as in *T. gondii*, most likely composed of a microneme (mics)-rhoptry neck protein (rons) complex. Following host cell invasion, parasites are enclosed in a parasitophorous vacuole, surrounded by a parasitophorous vacuole membrane (arrows in B.) Tachyzoite-dense granule components are secreted into the lumen of the parasitophorous vacuole (pv in 2C) and incorporate also into the vacuolar membrane (pvm marked by arrows in 2D). Dense granule components are also important in the formation of the parasitophorous vacuole tubular network (tn) that builds up the matrix between the parasites within the pv. Bars in A=4µm, B=1µm, C=3µm, D=2.5µm

host cell nucleus, and they become physically associated with mitochondria and the ER (53). 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 (18, 19, 54, 55; see also Figure 2C, D).

ROP proteins are discharged at the early phase of vacuole formation. *T. gondii* ROP2, one of the members of the ROP2-family, is inserted into the vacuole membrane during invasion and is exposed to the host cytoplasm, and mediates anchoring to host cell mitochondria (56). Targeted depletion of ROP2, resulted in multiple effects, including (i) impairment of rhoptry biogenesis and cytokinesis, (ii) reduction in the association of host cell mitochondria with the PVM of the parasite, (iii) reduced invasive and replicative capacities of the parasite, and (iv) attenuation of virulence in mice (57). Antibodies directed against TgROP2 inhibit invasion of human fibroblast by *T. gondii* in vitro (58). All members of the ROP2 family contain a kinase-like domain, but only some of them are active.

ROPs are involved in crosstalk and manipulation of host cell functions. Of these TgROP16 and TgROP18 act as hypervariable protein kinases that are responsible for high virulence of certain strains of *T. gondii*. This in contrast to TgROP2 and several other members of this family, that lost detectable kinase activity (56, 59). El Hajj (60) demonstrated that TgROP18 phosphorylated two parasite proteins, and TgROP18 overexpression in *T. gondii* resulted in increased proliferation. In contrast, TgROP16 is translocated to the host cell nucleus (61), subverts STAT3/6 signalling, and as a consequence IL12 production in infected host cells. Analysis of type I, II and III virulent *T. gondii* parasites showed that expression of TgROP16 correlated with high virulence strains. Thus, ROPs represent promising vaccine candidates.

4. VACCINES AGAINST *NEOSPORA CANINUM*

Considering the economic and agricultural impact of neosporosis, there is an urgent need to develop control measures aimed at preventing its transmission and infection, as well as reducing severity of the disease (15, 16). Basically, an efficient vaccine against *N. caninum* infection should fulfill the following requirements: (i) prevention of tachyzoite proliferation and dissemination in pregnant cattle (or other animals) to avoid transplacental transmission to the fetus; (ii) prevention or reduction of oocyst shedding in dogs (or other possible final hosts); (iii) prevention of tissue cyst formation in animals that have been infected with oocysts or tissue cysts (to avoid parasite transmission to carnivorous hosts).

This could be achieved by a vaccine that stimulates protective cellular immune responses (reviewed in 17, 62, 63, 64) as well as antibody responses (65) at both, mucosal sites and systemically. This is best achieved by delivering these antigens to antigen presenting cells (APCs) such as B cells and dendritic cells in a manner that allows optimal presentation via MHC class II molecules. However, experiments in cattle showed that antibody responses appear to be less important (64). In fact, cytotoxic T cells that kill *Neospora*-infected cells in cattle are, surprisingly, CD4+-positive (65). The T-helper cells activated by this vaccine must exert protective activities by the production of cytokines (IFN-gamma, TNF-alpha, IL12, or IL10 and IL4, depending whether a Th1- or Th2-type immune response is elicited) and cytokines such as IL-2. Specific antibodies that are generated could aid in limiting parasite proliferation by killing extracellular tachyzoites, e.g. by interference with host cell invasion, activation of complement, and/or opsonization of parasites to have them killed by macrophages. This multitude of tasks is very unlikely to be achieved by vaccination with a single, defined antigen, but most likely with a mixture of parasite antigens, or a vaccine that contains a number of relevant antigenic domains of different proteins.

In spite of the fact that rodents and cattle exhibit somewhat different immunological characteristics, studies using mice have led to a better understanding of the complex immune response that dictates *N. caninum* infection (14, 17, 21). Thus, studies in mice provide a "proof-of-concept-model" and also provide important information on the immuno-protection associated with intracellular parasitism *per se*. Protective immune responses against experimentally induced neosporosis in acute disease mouse models have been mainly associated with the development of a Th1-type immune response, dominated by IgG2a antibody production, and natural killer (NK) cell proliferation with increased IFN-gamma production (67).

However, there are also reports on protective effects achieved by Th2-type responses in acute disease (68, 69) and in fetal infection models (70). Thus, both Th1 and Th2-driven immune mechanisms can limit disease, at least in the mouse model.

4.1. Killed tachyzoite lysates / live-attenuated vaccines

The first hint that an approach employing a complex mixture of antigens could confer protection against neosporosis was provided by Liddel et al. (71), which showed that immunization with a killed tachyzoite lysate prevented fetal infection in mice. To date, a commercial vaccine based on killed tachyzoite lysate (Neoguard™) is currently available in the US, with uncertain efficacy data (62, 63). Clearly, protection against fetal infection with a killed tachyzoite lysate is not achieved in cattle, but the abortion rate was reduced by 24.6% in one study, and by more than 50% in another report (72). In contrast, Williams et al (64) reported complete protection against experimental *N. caninum* infection during pregnancy using a naturally attenuated *N. caninum* isolate (Nc Nowra)

The failure of a killed tachyzoite lysate to induce protection against infection in cattle could be related to the presence of a multitude of different *Neospora* antigens that exhibit detrimental antigenic properties. Some antigens, of course, when inoculated into an animal, will modulate the immune response in favor of the host, thus confer

protection, but others could also induce the opposite effect. This was demonstrated by one of our own studies, where mice were vaccinated with different formulations of a vaccine that was based on NcMIC4, a microneme protein identified in our laboratory (73). Application of (i) purified NcMIC4 in its native form, (ii) as a recombinant protein (recNcMIC4), and (iii) as a DNA vaccine actually induced increased mortality and increased cerebral infection upon challenge-infection in mice. The exact reasons for this are not known, but it is possible that such anti-protective antigens might not be exposed so efficiently to the immune system during in vivo infection, and/or their expression is more tightly regulated in attenuated isolates such as Nc Nowra (64).

The only vaccine against toxoplasmosis (currently licensed for use in sheep in Europe and NewZealand) is a live vaccine (Toxovac™), which contains live attenuated tachyzoites of the non-persistent strain *T. gondii* S48 (74). However, Toxovac™ does not protect against *N. caninum* infection and the vaccine has not been licensed in humans or other species because it is not well defined and there is the fear of reverting to tissue cyst formation. Nevertheless, others have taken over the option of vaccinating mice (75) or cattle (64) with live tachyzoites of the naturally attenuated low virulence *N. caninum* isolate Nc Nowra. Challenge of these animals with the virulent Nc-Liverpool isolate resulted in infection of fetuses in only 8% of cases in mice and in complete protection in cattle. This data is impressive and suggests that in case of achieving protection against *N. caninum* infection during pregnancy, live vaccines, such as for toxoplasmosis in sheep, will be the way forward. Others have generated temperature-sensitive mutants of *N. caninum*, or have applied irradiation of tachyzoites, thus treatments that have lead to suppressed proliferation. Therefore, these parasites did not cause disease in mice, but showed protective efficacy in the mouse model against experimental *N. caninum* infection (76, 77). However, the genotypes of all these parasite populations are not well defined, and the risks associated with using live parasite populations as live vaccines in cattle may be significant, and there is the concern that these organisms might reverse to a disease-causing phenotype. Thus, regulatory hurdles to get such a vaccine marketed are high.

In contrast to live vaccines, vaccines composed of recombinant proteins have distinct advantages, such as ease of manufacture, longer shelf life, ease in handling and application. It is possible that a focused selection of relevant antigens will also induce the relevant immune responses and reduce not only abortion, but also lead to reduced fetal infection rates.

We propose, that antigens that are exposed at the parasite or host cell surface, either constitutively or temporarily, represent suitable vaccine candidates. For a proof of principle, these antigens can be conveniently investigated in the murine model, prior to embarking on costly experiments involving cattle.

4.2. Components of the host cell adhesion/invasion machinery of *N. caninum* and related apicomplexans represent potential vaccine targets

A limited number of recombinant proteins have been investigated as vaccine candidates against neosporosis. These include mostly immune-dominant antigens functionally involved in tachyzoite-host cell interactions. The surface antigen NcSRS2 expressed in recombinant vaccinia virus offered adequate protection against transplacental passage and was found to limit parasite dissemination (78). Immunization of mice against neosporosis with different recombinant NcSRS2 iscom formulations was reported to induce specific antibody responses to native NcSRS2, and a significant reduction of cerebral parasite load in immunized mice (79, 80). In fact, NcSRS2 in its native form was shown to protect against *N. caninum* congenital transmission in mice, by eliciting a Th2-type immune response (70). Reduced materno-fetal transmission was seen upon vaccination with NcGRA7 as well as NcHSP33 as a DNA vaccine (81). Further, the immune protective effect of NcGRA7 was enhanced upon use of it as a plasmid DNA together with CpG adjuvant, which improved the protective efficacy against fetal infection (82). Cho et al. (83) reported vaccination efficacy in an acute disease model upon combining recombinant NcSRS2 and dense granule antigen NcDG1 (=NcGRA7). Aguado-Martínez *et al.* (84) employed recombinant NcGRA7 (expressed in both tachyzoite and bradyzoite stages) and NcSAG4 (a major *N. caninum* bradyzoite antigen) as vaccines in the fetal infection model and this did not result in notable protection of offspring against transplacental infection or disease. Application of recombinant NcMAG1, a dense granule antigen that is increasingly expressed in the cyst matrix upon tachyzoite-to-bradyzoite differentiation (85) as a vaccine resulted in 50% of mice protected against clinical signs (86).

Expression of the *N. caninum* antigens NcMIC1, NcSRS2, NcGRA7 and NcGRA6, respectively, in the *Brucella abortus* vaccine strain RB51 and subsequent immunization of mice resulted in complete protection against experimentally induced acute disease (87), and NcGRA6 and NcMIC1 expressed in *Brucella abortus* protected against vertical transmission (88). Ellis et al. (76) reported a very limited protection against transplacental transmission of *N. caninum* in mice following application of MIC10 and p24B recombinant antigens, but no protection at all using recombinant versions of NGRA1, NcGRA2, MIC10 and p24B alone.

Our own studies on *N. caninum* vaccines have initially, focussed on major immunodominant tachyzoite antigens. This included the major surface antigens NcSAG1 (89), and NcSRS2 (90), the dense granule antigen NcGRA7 (91), and the microneme antigen NcMIC3 (30, 35, 36). The latter two are also expressed in bradyzoites. Fuchs et al (92) showed that *N. caninum* and *T. gondii* tachyzoites exhibit considerable differences with regard to the presence of surface glycoconjugates, and one of the *N. caninum* antigens that binds to the lectin Con A is the microneme protein NcMIC1 (29). Antibodies directed against NcMIC1 severely inhibit parasite adhesion to the host cell surface

(93). Another microneme protein, which itself acts as a lectin is NcMIC4, which binds to chondroitinsulfate A (38, 73). Protein disulfide isomerase (NcPDI) was also found to be partially localized within micronemes, but also on the surface of *N. caninum* tachyzoites (94). Incubation of *N. caninum* tachyzoites with affinity-purified anti-NcPDI, anti-NcMIC4 and anti-NcMIC1 antibodies reduced host cell adhesion, implying that these antibodies interfere with parasite-host cell binding (94, 95). Another class of proteins involved in host cell invasion are rhoptry proteins such as NcROP2: The NcROP2 sequence shares 46% identity with *T. gondii* ROP2, and like other members of the ROP2 family, it contains a serine/threonine protein kinase domain (authors, unpublished observations).

The protection mediated by these antigens was evaluated in the mouse model that allows monitoring of acute disease. Mice vaccinated with individual recombinant recNcSRS2 and recNcSAG1 did not exhibit high levels of protection against challenge infection with *N. caninum* tachyzoites (30-40%), except when a combined protocol utilizing DNA vaccination and subsequent boost with the corresponding antigens was performed (75% for NcSRS2; 62.5% for NcSAG1) (96).

Most promising results in terms of protection against cerebral disease and cerebral parasite load were achieved with the two micronemal antigens NcMIC1 (93) and NcMIC3 (68), and with the rhoptry antigen recNcROP2 (97, 98). Mice vaccinated with recNcMIC3 antigen exhibited high percentage (75%) of protection against cerebral infection (68). Protection was associated with an IgG1 antibody response (thus possibly Th2-biased). A DNA vaccine-approach did not result in any significant protection (unpublished findings). Upon recNcMIC1-vaccination and subsequent challenge infection, all mice were PCR positive in the brain, but with very low parasite burden. RecNcMIC1-vaccinated mice did not show any clinical signs of disease (93). In contrast, DNA vaccination was not protective. Following challenge with *N. caninum* tachyzoites, mice vaccinated with recNcROP2 remained clinically healthy and exhibited highly significantly reduced cerebral parasite burden. IgG-isotype ELISA indicated that NcROP2 induces a protective Th-1- or Th-2-biased immune response against experimental *N. caninum* infection, depending on the adjuvants (Freunds versus Saponin) used for immunization (97). The fact that some antigens can confer anti-protective effects was demonstrated by vaccination of mice with NcMIC4-based vaccines and subsequent challenge. Application of native, purified NcMIC4, recNcMIC4 and a NcMIC4-DNA-vaccine resulted in increased cerebral parasite burden and mortality (73).

The protective potential of recombinant his-tagged antigens recNcMIC1, recNcMIC3 and recNcROP2 was investigated in Balb/c mouse models for cerebral and fetal infection (98). Mice were vaccinated with recNcROP2, and combined recNcROP2/NcMIC1, recNcROP2/NcMIC3, and recNcROP2/NcMIC1/NcMIC3 antigens, all resuspended in saponin adjuvants. Subsequently, mice were mated, and challenge-infected. Those dams receiving either recNcROP2 or the recNcROP2/NcMIC1/NcMIC3-combination vaccine did not exhibit any morbidity, while 50% and 43% of mice succumbed to disease in the infection control and adjuvant control groups, respectively. For pups, the highest survival rates were noted for the groups receiving recNcROP2 (50%) and recNcROP2/NcMIC1/NcMIC3 (35%), while in the infection- and adjuvant-control groups all pups died latest at days 25 and 30, respectively. Quantification of parasite DNA by *N. caninum*-specific real time PCR revealed consistently lower parasite burdens in brain tissue of pups from vaccinated groups compared to the controls. However, GRA2 real time RT-PCR on brain tissue of surviving pups — applied to detect viable parasites — demonstrated that only the pups from the group vaccinated with the recNcROP2/NcMIC1/NcMIC3 combination appeared free of viable tachyzoites, while in all other groups viable parasites were still present. Serological analysis of humoral (total IgG, IgG1, IgG2a) and serum cytokine (IL-4, IFN-g) responses showed that this effect was associated with a Th-2-biased immune response, with a clearly elevated IL-4/IFN-g ratio in the mice receiving all three antigens in combination. Thus, a mixture of recombinant antigens representing important secretory micronemal and rhoptry proteins leads to a significant Th2-biased protection against vertical transmission of *N. caninum* in mice (98).

In another series of experiments applying the acute disease mouse model, Debache et al (86) demonstrated that the efficacy of some vaccines is dramatically altered depending on the route of antigen delivery. *E. coli*-expressed recombinant NcPDI(recNcPDI), NcROP2(recNcROP2), and NcMAG1(recNcMAG1) were evaluated as potential vaccine candidates by employing the murine cerebral infection model. Intra-peritoneal application of these proteins suspended in saponin adjuvants lead to protection against disease in 50% and 70% of mice vaccinated with recNcMAG1 and recNcROP2, respectively, while only 20% of mice vaccinated with recNcPDI remained without clinical signs. In contrast, a 90% protection rate was achieved following intra-nasal vaccination with recNcPDI emulsified in cholera toxin. Thus, when applied intranasally, recNcPDI exhibits highly protective properties. Only one mouse vaccinated intra-nasally with recNcMAG1 survived the challenge infection, thus NcMAG1 has lost its protective properties entirely, and protection achieved with intra-nasally applied recNcROP2 was the same, no matter which route of antigen delivery was applied. Determination of cerebral parasite burdens by real time PCR showed that these were significantly reduced only in recNcROP2-vaccinated animals (following intra-peritoneal and intra-nasal application) and in recNcPDI-vaccinated mice (intra-nasal application only). Quantification of viable tachyzoites in brain tissue of intra-nasally vaccinated mice showed that immunization with recNcPDI resulted in significantly decreased numbers of live parasites. These data show that, besides the nature of the antigen, the protective effect of vaccination also depends largely on the application mode. In the case of recNcPDI, the intra-nasal route provides a platform to generate a highly protective immune response.

5. WHERE TO GO FROM HERE

The authors of this review and others hypothesize that antigenic molecules associated with proteins involved in adhesion/invasion or other host-cell interaction processes represent important targets for vaccination against *Neospora caninum* infection. Thus, we postulate that the development of a vaccine can possibly be achieved by employing recombinant subunit vaccines. However, if one evaluates the data available today, even highly optimistic minds must conclude that there is a lot of room for improvement.

Not many non-live subunit vaccines have been assessed as vaccines for the prevention of congenital transmission of *N. caninum* and fetal death so far. The most successful trial was reported by Haldorson et al. (70), which showed that vaccination of Balb/c mice with affinity-purified native NcSRS2 resulted in reduced congenital transmission that was associated with a high IL-4/IFN-g ratio in splenocytes stimulated with native NcSRS2. Based on the very good protection rates achieved by live vaccination with the naturally attenuated NC-Nowra isolate, and the relatively modest success employing recombinant vaccines, Ellis et al. (76) concluded that live vaccines will be the way forward, similar to those vaccines currently available for besnoitiosis in cattle (99), *Toxoplasma* induced abortion in sheep (100) and coccidiosis in poultry (101). However, live vaccines have some disadvantages in terms of safety, costs of production, and stability of the final product. In addition, it will not be possible to distinguish between infected and vaccinated animals (102). Subunit vaccines have clear advantages such as reduced costs in production, processing and storage, increased stability and shelf life.

Thus, in order to go on, we need to address some important aspects. First, various authors have reported conflicting results with homologous antigens in *Toxoplasma* and *Neospora* vaccination experiments (17, 21). Therefore, it is important to elucidate what *Neospora* is doing differently compared to *Toxoplasma*, and how we could exploit these differences to generate an efficient vaccine (or any other tools of intervention). Clearly, a major drawback (although others would say it is a highly valuable advantage) in *Neospora* research has been the fact that we have largely extrapolated the current knowledge on the cell biology, and especially the molecular aspects of host-parasite interactions, obtained from other apicomplexan models such as *Toxoplasma* to the situation in *Neospora*. In many cases this has been done without carefully evaluating potential differences between the two species. Clearly, more basic, and independent research on the molecular nature and function of selected *Neospora* antigens is needed, in order to evaluate their true usefulness as potential vaccine candidates. Secondly, the study by Debache et al. (86) illustrates how the formulation of an antigen and its route of delivery can alter its protective properties. Thus, how should antigens be formulated, and how should these formulations modulate/enhance the immune response? Since different groups employ different animal models, routes of infection and employ different parameters for assessment of vaccines, it is difficult to directly compare the results coming from different laboratories, and a certain level of standardization would represent a great improvement. Thirdly, there is a great need for the development of a laboratory recrudescence model, which would mimic the situation of endogenous transplacental infection as it occurs in cattle in the field. However, there are admittedly substantial differences in the inherent properties of the immune responses between mice and cattle, and any results derived in a small animal laboratory model need to be evaluated with caution.

Recombinant subunit vaccines have been developed against other parasitic diseases and some of these are highly effective in preventing infection with *Taenia ovis* in sheep, *Taenia saginata* in cattle, *Taenia solium* in pigs and *Echinococcus granulosus* in livestock animals (reviewed in 103). *T. ovis* and *T. saginata* are economically significant parasites and the commercial success of vaccines against them relies on their economic value. In the case of neosporosis, the economic value is given, and what is needed is a safe and effective subunit vaccine.

Finally, how good is our hypothesis that emphasizes the usefulness of antigens involved in host cell invasion as vaccines? Current studies as shown above indicate that certain antigens that are involved in the physical interaction between parasite and host cell exhibit a good potential to mediate protection. However, the life cycle of *N. caninum* is complex, and it make sense to incorporate other antigens, into the current arsenal of potential vaccine targets. Thus, we propose that the search for promising antigens must go on, and the ideal recombinant vaccine against neosporosis should be composed of different subunits, which complement each other in conferring efficient protective immunity.

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