Towards a preventive strategy for neosporosis: challenges and future perspectives for vaccine development against infection with *Neospora caninum*

Yoshifumi NISHIKAWA

1) National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

**Abstract.** Neosporosis is caused by the intracellular protozoan parasite *Neospora caninum*. This major disease-causing pathogen is responsible for inducing abortion in cattle, and these adverse events occur sporadically all over the world, including Japan. Currently, there are no vaccines on the market against infection with *N. caninum*. Because live and attenuated vaccines against *N. caninum* have had safety and effectiveness issues, development of a next-generation vaccine is urgently required. To develop a vaccine against neosporosis, my laboratory has been focused on the following: 1) understanding the host immune responses against *Neospora* infection, 2) identifying vaccine antigens and 3) developing an effective antigen-delivery system. The research strategy taken in my laboratory will have strong potential to progress current understanding of the pathogenesis of *N. caninum* infection and promote development of a novel subunit vaccine based on the specific vaccine antigen with an antigen-delivery system for controlling neosporosis.

**KEY WORDS:** adjuvant, cattle, *Neospora caninum*, neosporosis, vaccine

*Neospora caninum* is an intracellular apicomplexan protozoan parasite with a close relationship to *Toxoplasma gondii* [5]. Since its first recognition in dogs in Norway in 1984 [4] and the description of it as a new genus and species [8], neosporosis from *N. caninum* has emerged as a serious disease in cattle and dogs worldwide. The *N. caninum* life cycle can be classified broadly into three stages: tachyzoites, bradyzoites (tissue cysts) and sporozoites (oocysts) (Fig. 1). The definitive hosts of *N. caninum* are canids, including dogs, dingoes, gray wolves and coyotes, but not foxes [10, 19, 21]. A variety of animals ranging from birds to mammals including cattle, sheep, horses and deer can act as its intermediate hosts [5, 7]. *N. caninum* can be spread by horizontal transmission of the oocysts shed by the definitive hosts and vertical transmission via the placenta in pregnant animals [5, 7]. When infected with oocysts, the sporozoites released from the gastrointestinal tract wall of the host spread throughout the whole body via parasite conversion from sporozoites to tachyzoites. Although the host immune response controls tachyzoite proliferation at each infection site, the parasites escape from the host immunity in the central nervous system and muscle tissues, resulting in them transforming into bradyzoites [22]. The bradyzoites then slowly multiply to form tissue cysts, which are another source of *Neospora* infection when they are orally ingested by another susceptible animal. Immunocompromised hosts develop severe neosporosis, while immunocompetent animals generally show no obvious clinical signs of infection. Abortion, stillbirth and neurological disease associated with *N. caninum* infection cause major economic problems in the livestock industry worldwide [6], and *Neospora*-infected cattle cannot be used for milking [6]. Vertical transmission of *N. caninum* infection can be maintained over several generations, a major factor for the expansion of the infection [41]. In fact, *Neospora* infections have a worldwide distribution, which includes Japan, and the global economic impact of this infection has been reported [40]. The total annual cost of *N. caninum* infections and abortions is estimated to range from a median US$1.1 million in the New Zealand beef industry to an estimated median total of US$546.3 million impact per annum in the USA dairy population. The estimate for the total median losses for *N. caninum* infections exceeds US$1.298 billion per annum, ranging as high as US$2.380 billion. Therefore, because vaccines against infection with *N. caninum* are not currently available, development of a safe and effective vaccine is urgently needed.
To develop an effective vaccine against *N. caninum*, systematic research on host-immune responses against this parasite is required. We can use experimental mice to analyze the immune responses and the pathology caused by this parasitic infection. In fact, parasite infiltration of the brain, lungs, liver, spleen and heart is all detectable, and reproducible results for the neurological signs and vertical transmission of the parasite are obtainable in mice [1, 12, 14, 29, 35]. Previous studies have shown that humoral and cellular immunities including a balance of T-helper 1 (Th1) and T-helper 2 (Th2) activity are required for controlling *N. caninum* infection [26, 27]. Interactions between the host cell surface and parasite protein(s) may play crucial roles during invasion of *N. caninum* into host cells. One of the candidates involved in the *N. caninum* invasion process is NcSRS2, a surface antigen of the parasite. Monoclonal antibodies (mAbs) against NcSRS2 partially inhibit *N. caninum* invasion of host cells [33].

The Th2-type immune response, with predominant production of humoral antibodies specific for parasite antigens, is capable of mediating protection against neosporosis [11]. However, transferring antibodies against NcSRS2 to mice does not inhibit the vertical transmission of *N. caninum* [28]. These results suggest that the reactions of specific antibodies alone cannot provide effective control of *N. caninum* infections. Because *N. caninum* exists in the host cell cytosol, the antibodies are not able to bind to the parasites. Nonetheless, because cellular immunity can destroy pathogen infected cells via cytotoxic T cells, this type of host immunity will contribute to the elimination of *N. caninum*-infected host cells. Interferon-gamma (IFN-γ) and interleukin-12 (IL-12), both of which are crucial cytokines for the development of Th1-type immunity (cellular immunity), are important for obtaining protective immunity against acute *N. caninum* infections. In the case of infection with *N. caninum*, the production of IL-12 and IFN-γ is detectable in host animals. Mice deficient in the IL-12 gene or the IFN-γ gene are susceptible to *N. caninum*.
Infection with *N. caninum* 

![Infection with N. caninum](image)

Induction of parasiticide-specific CD4+ and CD8+ T cells

 Activation of antigen-presenting cells 

![Activation of antigen-presenting cells](image)

Macrophages or dendritic cells (antigen-presenting cells)

CD4+ T cells

CD8+ T cells

Elimination of *N. caninum*-infected cells by parasite-specific CD4+ and CD8+ T cells

Nitric oxide-dependent killing

![Elimination of N. caninum-infected cells by parasite-specific CD4+ and CD8+ T cells](image)

Fig. 2. Protective immunity against infection with *N. caninum*. After becoming infected with *N. caninum*, antigen-presenting cells (APCs), such as macrophages and dendritic cells, phagocytose the parasite or parasite-derived antigens. Then, activation of APCs triggers induction of parasiticide-specific CD4+ and CD8+ T cells via IL-12 production from the APCs to eliminate the *N. caninum*-infected cells. IFN-γ produced from the activated T cells stimulates nitric oxide production, and this can kill the intracellular parasites.

Infection [25, 31], indicating the importance of these cytokines in host protective immunity. In addition, macrophages play an important role in the cellular effector responses that reduce tissue parasitism and enhance host survival, because nitric oxide production by macrophages treated with IFN-γ can control the growth of *N. caninum* [31]. Moreover, macrophages can produce IL-12, a key cytokine linking the innate and adaptive compartments of the immune system. The normal production of IL-12 is initiated through recognition of highly conserved sets of molecular patterns (pathogen-associated molecular patterns, PAMPs) through pattern-recognition receptors (PRRs) [18]. However, the interaction of these PAMPs with PRRs on innate cells triggered by *N. caninum* infection has not yet been fully elucidated. Previous studies showed that engagement of Toll-like receptor 2 (TLR2), TLR3 and TLR11 triggered activation of the myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathways [3, 16, 23]. The CCR5 chemokine receptor is also a key player in the immune response against *N. caninum*, because mice deficient in the CCR5 gene are susceptible to the infection [1]. Additionally, the interaction of CCR5 with parasite-derived cyclophilin triggers migration and activation of innate cells [17, 24]. Thus, MyD88- or TRIF-dependent pathways through TLRs or chemokine receptors can stimulate host immune responses against infection with *N. caninum*.

Based on these observations, the important steps in protective immunity against infection with *N. caninum* are as follows (Fig. 2):

1. Activation of antigen-presenting cells, such as macrophages and dendritic cells.
2. Elimination of *N. caninum*-infected cells by parasite-specific CD4+ and CD8+ cells.
3. Nitric oxide-dependent killing of *N. caninum* by the IFN-γ produced by activated immune cells.

However, proinflammatory cytokines, such as IFN-γ, may also trigger severe pathology in the host. Thus, these observations suggest that a suitable balance in the induction of Th1/Th2-type immunity is crucially important to the effective control of *N. caninum* infection [27].

**VACCINE DEVELOPMENT AGAINST N. CANINUM INFECTION**

To design an effective vaccine against *N. caninum* infection, the induction of both Th1- and Th2-type immunity should be considered. For cattle, the importance of Th1-type cellular immunity has been reported as necessary for the development of
Developing an Antigen Delivery System

Identification of Vaccine Antigens

Antigens, after which they are selected by their inhibitory effects on immunizing mice with recombinant vaccinia virus expressing NcSRS2, induced cellular immunity against and the pathways to generate antigen-specific CTLs and Th1 cells, respectively, and lipid antigen to CD1d to activate natural killer T cells. In addition, OMLs can also deliver encapsulated antigens to the major histocompatibility complex (MHC) class I and MHC class II phagocytic cells secrete IL-12 selectively and enhance the expression of costimulatory molecules, indicating their adjuvant activity.

Therefore, the development of a safe and effective subunit vaccine for controlling N. caninum infection should be of high priority. The next subjects of discussion for Neospora vaccine development are as follows: 1) identifying novel antigens capable of inducing protective immunity against neosporosis and 2) developing a novel antigen delivery system.

Identification of Vaccine Antigens

My laboratory’s strategy for identification of N. caninum vaccine antigens is as follows:

1. Immune screening of N. caninum cDNA libraries using parasite-specific mAbs and antigen-specific polyclonal antibodies [33, 46]. The mAbs and polyclonal antibodies are generated by injecting mice with live parasites, parasite lysates or specific antigens, after which they are selected by their inhibitory effects on N. caninum invasion of host cells. N. caninum cDNA libraries are screened with the selected antibodies via incubation on nitrocellulose sheets containing phage plaques. Positive plaques are visualized using an enzyme detection system (e.g., an alkaline phosphate-conjugated antibody with a suitable substrate). The cloned insert in the plaque-purified λ phage is then subcloned into a plasmid vector for cDNA sequencing.

2. Immune proteomics of N. caninum antigens using parasite-specific anti-sera [43, 46]. For target antigen determination, N. caninum lysates are separated using sodium dodecyl sulfate-poly acrylamide gel electrophoresis. A two-dimensional gel electrophoresis (2-DE) and protein identification by mass spectrometry are performed. The proteins separated by 1-DE and 2-DE are visualized with colloidal Coomassie brilliant blue or are transferred onto a membrane for immunoblotting. After spot image analysis, the trypsin-digested peptide mixture from the protein spot is analyzed using MALDI-TOF mass spectrometry.

3. Screening N. caninum antigens using enzyme-linked immunosorbent assay (ELISA) with recombinant antigens and field sample-obtained sera [13]. The ELISA is established using N. caninum recombinant proteins expressed in Escherichia coli. The reactive antigens are detected using field samples of anti-sera from N. caninum-infected cattle, an important step in vaccine development against bovine neosporosis. Moreover, this approach makes correlation analysis between specific-antibody positive animals and abortion possible.

However, identifying antigens using the above-mentioned methods requires specific antibodies. Therefore, the efficacy of cellular immunity induction should be considered by further analyses. In our studies, the reactivity of candidate antigens is tested by T-cell responses using spleen cells or lymphocytes from N. caninum-infected animals [29, 36].

Development of an Antigen Delivery System

In studies on Neospora vaccine development, several types of vector, such as vaccinia virus [30, 34], canine herpesvirus [32] and the Brucella abortus RB51 strain [38, 39, 42] have been tested as antigen delivery systems. Our previous study showed that immunizing mice with recombinant vaccinia virus expressing NeSRS2, induced cellular immunity against N. caninum infection. Especially, the immunizations reduced the cerebral infections and prevented vertical transmission of N. caninum in mice [30, 34]. These results suggest that a non-infectious antigen-delivery system that can induce cellular immunity should be efficacious.

Although virus or bacteria-based vectors can induce strong immunity, the safety of the vectors may be problematic. Therefore, the development of a safe antigen delivery system lacking a live pathogen vector is urgently needed. In one of our studies, we focused on developing a next-generation vaccine based on liposomes coated with neoglycolipids that contain oligomannose residues (OMLs) as a novel adjuvant for the induction of Th1 immune responses and cytotoxic T lymphocytes (CTLs) specific for the encased antigen [9]. The liposomes were coated with a neoglycolipid consisting of mannnotriose and dipalmitoylphosphatidylethanolamine (otherwise called Man3-DPPE) [20] (Fig. 3). OMLs preferentially take up peripheral phagocytic cells and migrate into lymphoid tissues from the peripheral tissues. Importantly, in response to OML uptake, the phagocytic cells secrete IL-12 selectively and enhance the expression of costimulatory molecules, indicating their adjuvant activity. In addition, OMLs can also deliver encapsulated antigens to the major histocompatibility complex (MHC) class I and MHC class II pathways to generate antigen-specific CTLs and Th1 cells, respectively, and lipid antigen to CD1d to activate natural killer T cells. Thus, OMLs entrapped in Neospora antigen have potential as a novel vaccine against N. caninum infection.

My laboratory has identified dense granule protein 7 (NcGRA7) and apical membrane antigen 1 (NcAMA1) of N. caninum as candidate vaccine antigens [13, 45], and generated OMLs entrapping these antigens. The OML-entrapped NcGRA7 and NcAMA1 induced N. caninum-specific cellular and humoral immunity, and prevented the vertical transmission of the parasite in mice.
Moreover, cellular and humoral immunity against *N. caninum* and reduced cerebral infections were observed in cattle immunized with the OML-entrapped NcGRA7 [36]. Therefore, OML can be used as a novel and effective vaccine for controlling vertical transmission and abortion from bovine neosporosis.

**FUTURE DEVELOPMENTS**

Previous studies have revealed the different aspects of the host–parasite relationship in pregnant cattle [15]. When cattle are experimentally infected with *N. caninum* early in gestation, death of the fetus can occur. Early in gestation, the mother is able to induce a strong cell proliferation response with production of IFN-γ against *N. caninum*. Therefore, abortion related to *N. caninum* infection may be caused by inflammation at the maternal–fetal interface, because the maternal immune responses, such as T cell reactions against infection with *N. caninum*, are induced. However, significant immunomodulation of the immune response occurs at mid-gestation, and this may trigger reactivation of the parasites in persistently infected cattle. Although the Th2 cytokine environment at the maternal–fetal interface is maintained at mid-gestation, it cannot control the *N. caninum* infection, and parasite invasion of the placenta and infection of the fetus occur. At this gestational stage, the parasite infection can trigger the death of the fetus, or the calf, both of which are congenitally infected and show some clinical signs, and they may be born as a result of vertical transmission of the disease. In late gestation, the fetal immune system matures, and it can control the infection. Therefore, the calf is congenitally infected, but otherwise healthy. Thus, the gestational age of the fetus at the time of infection is crucial in determining the severity of neosporosis.

According to what is known about the host–parasite relationship in pregnant cattle, vaccination of such cattle is not realistic. Therefore, vaccinating cattle before pregnancy is a more realistic goal for controlling neosporosis. The next objective is to evaluate the protective efficacy of the OML vaccine on abortion and vertical transmission in cattle induced by *N. caninum* infection. This research strategy will have strong potential to progress current understanding of the pathogenesis of *N. caninum* infection and promote development of a novel vaccine for controlling neosporosis. Use of this approach will shed new light on the basic biology of *N. caninum* and advance *Neospora* research relating to the activities and applications of science to clinical disease.

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