Vaccination as a control strategy against the coccidial parasites *Eimeria*, *Toxoplasma* and *Neospora*

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SUMMARY

The protozoan parasites *Eimeria* spp. *Toxoplasma gondii* and *Neospora caninum* are significant causes of disease in livestock worldwide and *T. gondii* is also an important human pathogen. Drugs have been used with varying success to help control aspects of these diseases and commercial vaccines are available for all three groups of parasites. However, there are issues with increasing development of resistance to many of the anti-coccidial drugs used to help control avian eimeriosis and public concerns about the use of drugs in food animals. In addition there are no drugs available that can act against the tissue cyst stage of either *T. gondii* or *N. caninum* and thus cure animals or people of infection. All three groups of parasites multiply within the cells of their host species and therefore cell mediated immune mechanisms are thought to be an important component of host protective immunity. Successful vaccination strategies for both *Eimeria* and *Toxoplasma* have relied on using a live vaccination approach using attenuated parasites which allows correct processing and presentation of antigen to the host immune system to stimulate appropriate cell mediated immune responses. However, live vaccines can have problems with safety, short shelf-life and large-scale production; therefore there is continued interest in devising new vaccines using defined recombinant antigens. The major challenges in devising novel vaccines are to select relevant antigens and then present them to the immune system in an appropriate manner to enable the induction of protective immune responses. With all three groups of parasites, vaccine preparations comprising antigens from the different life cycle stages may also be advantageous. In the case of *Eimeria* parasites there are also problems with strain-specific immunity therefore a cocktail of antigens from different parasite strains may be required. Improving our knowledge of the different parasite transmission routes, host-parasite relationships, disease pathogenesis and determining the various roles of the host immune response being at times host-protective, parasite protective and in causing immunopathology will help to tailor a vaccination strategy against a particular disease target. This paper discusses current vaccination strategies to help combat infections with *Eimeria*, *Toxoplasma* and *Neospora* and recent research looking towards developing new vaccine targets and approaches.

Key words: *Eimeria*, *Toxoplasma*, *Neospora*, vaccination.

INTRODUCTION

The coccidial parasites *Eimeria*, *Toxoplasma* and *Neospora* are a significant cause of disease in food-producing animals worldwide with *Toxoplasma gondii* also being an important human pathogen. Treatment with drugs has been a significant control strategy, in particular to help combat avian coccidiosis. However, major challenges in recent years have been the development of drug resistance, the high cost of prophylactic drug treatment, in particular in developing countries, and increasing consumer concerns about drug residues in food. The search for new drugs has looked at examining biochemical differences between parasites and their hosts to identify new targets (reviewed by Coombs and Muller, 2002). A recent example is the discovery of the shikimate pathway in *Toxoplasma* and *Plasmodium* parasites which is important in the synthesis of aromatic compounds and folates. As mammals lack this pathway there is an opportunity to develop an intervention strategy based on selective inhibition of these enzymes. Increasing our knowledge of the biochemistry of many apicomplexan parasites by exploiting new technologies such as genomics and proteomics may facilitate the identification of potential targets common to this group of organisms providing some novel and effective broad-spectrum therapeutic agents. An alternative, and potentially more sustainable strategy which also addresses public concern about drugs in food animals, is the use of vaccination to control these diseases. While many drugs have broad-spectrum activity and can be active against several different parasite species, vaccines have to be customised to the particular parasite and sometimes to the particular host species. This requires considerable knowledge of the parasite biology, host-parasite interaction, different life cycle stages, antigens critical for parasite survival in the host and an understanding of the important components of protective immunity and how they are induced. There are commercial vaccines available to help protect against some of the diseases caused by these parasites. In this paper we will review work in

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this area, discuss the advantages and drawbacks of the existing vaccines and look at future directions and challenges in disease control.

EIMERIA

Eimeriosis, often designated as coccidiosis, is the disease caused by *Eimeria* parasites resulting in severe mucosal damage, weight loss and sometimes even death. The disease is widespread and many species are found in poultry, livestock and small animals such as rabbits. In poultry, clinical disease is caused by several species of *Eimeria* parasites including, *E. tenella*, *E. necatrix*, *E. brunetti*, *E. maxima*, *E. acervulina* and *E. mitis* (Long and Horton-Smith, 1968). A study examining the incidence in poultry in the Netherlands found *E. acervulina* and *E. tenella* in 63% of 4774 flocks examined (Graat *et al*. 1996). Under more natural circumstances the hosts and parasites are able to co-exist without too much difficulty, however, the intensification in poultry production (broiler farms) involving high stocking densities and restrictive habitats has resulted in coccidioses becoming a major disease problem (Vermeulen, 2004).

The life cycle of *Eimeria* parasites involves ingestion of sporulated oocysts, which excyst in the small intestine releasing sporozoites that infect the villar epithelial cells. The sporozoites then transform into trophozoites and undergo asexual multiplication becoming first generation schizonts forming numerous merozoites. The merozoites then lyse out of the cells and infect new epithelial cells and a second schizogony phase takes place. Merozoites then mature to form macrogametes and microgametes and a sexual cycle takes place resulting in the production of environmentally resistant oocysts which are shed in the faeces (Long and Horton-Smith, 1968). The life cycle of the different poultry *Eimeria* species are largely similar but may be distinguished by comparing morphology, number of schizogony cycles, location within the gut and period of oocyst shedding (Rose, 1996).

Immune mechanisms in avian coccidiosis

Since chickens readily develop immunity from natural infection (Horton-Smith *et al*. 1963) there was a good prospect to control the disease through vaccination. The immunity induced following infection with avian *Eimeria* is species specific (Rose, 1982) and in addition genetic diversity has been demonstrated within strains (Karim, Begum and Khan, 1994). Parasite stages that are thought to be important in generating protective immune responses include the initial asexual developmental stages where trophozoites multiply within the epithelial cells of intestinal villi (Jenkins *et al*. 1991). As is the case with other intracellular pathogens, T cells and cytokines are known to play an important role in protective immunity to *Eimeria*. Work done using rodent models of infection has shown that adoptive transfer of lymphocytes would protect against *E. vermiformis* (Rose *et al*. 1988) and depletion studies highlighted a role for CD4+ T cells in the induction of immunity and CD8+ T cells in the effector function following challenge (Rose, Hesketh and Wakelin, 1992). Studies using chickens have also highlighted the importance of cell mediated immunity in host protection as removal of the bursa did not affect the ability of the animal to generate a protective immune response (Rose and Long, 1970; Lillehoj, 1987). Adoptive transfer of immunity using immune spleen cells (Rose and Hesketh, 1982) and the increased susceptibility of chickens following treatment with agents designed to suppress cellular immune responses (Lillehoj, 1987) provided further evidence of the importance of cell mediated immunity in this disease. Vervelde, Vermeulen and Jeurissen (1996) and Breed *et al*. (1997) demonstrated the role of CD4+ and CD8+ T cells in chickens during the development of immunity after primary and secondary infection with *E. tenella*. In addition, such a role was confirmed by depletion of CD8+ T cells, which led to an increase in oocyst shedding (Trout and Lillehoj, 1996). Immune cytokines are known to be important in protective immunity to *Eimeria* (Ovington, Alleva and Kerr, 1995) and treatment of chicken cells with recombinant IFNγ inhibited growth of intracellular parasites (Lillehoj and Choi, 1998). There is a wide range of cytokines and chemokines induced following infection with *Eimeria* and work is progressing in identifying and characterizing chicken cytokine genes (Kaiser, Hughes and Bumstead, 1999) which will enable a better understanding of how the host responds to infection and how this may be exploited in developing more effective immunisation strategies. While Th1-type cytokines are likely to play an important role in limiting parasite multiplication in the early stages of infection, regulatory cytokines and other immune cells may play a vital role in limiting the immunopathology associated with pro-inflammatory cytokines. Interestingly, $\gamma\delta$ T cells form a large component of the intraepithelial lymphocyte population (Lillehoj, 1994; Vervelde *et al*. 1996) and have been shown to play an immunoregulatory role in helping to damp down the immunopathology caused by $\alpha\beta$ T cells and pro-inflammatory cytokines (Roberts *et al*. 1996). An understanding of how the immune system is activated and regulated is essential in enabling the design of novel vaccines and vaccination strategies. Very detailed studies of host immune responses to *Eimeria* are becoming possible using DNA microarray analysis (Min *et al*. 2005).

The anti-parasite effect of antibodies in *Eimeria* infection is not as well defined as cell mediated

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immunity. However, passive transfer of immunity has been reported using immune serum (Long and Rose, 1965), monoclonal antibodies (Crane *et al*. 1988) or hyperimmune serum directed against a gametocyte surface antigen (Wallach *et al*. 1992). Antibodies are likely to be active against extracellular parasites (Rose, 1996). A novel approach involved feeding chickens with egg antibody (IgY) powder, prepared from hens hyperimmunised using a defined recombinant protein of *Eimeria*. The chickens were protected from an oral challenge with oocysts indicating that passive immunisation through feeding antigen-specific IgY powder was a feasible approach (Lillehoj, 2005).

Approaches to vaccination

Live vaccines: Perhaps due to the importance of cell mediated immune responses in protection against *Eimeria*, live vaccination approaches, where parasite antigens are processed and presented to the immune system in the correct MHC context, have proved to be effective. Live vaccines may comprise virulent, wild-type parasites or attenuated, precocious laboratory strains. These vaccines are usually delivered orally in water and feed. Vaccines comprising virulent organisms of the most frequently occurring species, *E. tenella*, *E. acervulina* and *E. maxima*, rely on the administration of low doses of oocysts early in life. However, this has to be carefully administered as birds that do not ingest the vaccine are vulnerable to subsequent infection (Shirley, 1992). Attenuated strains of *Eimeria* that are not as pathogenic in the host but still confer protective immunity are also an effective option for live vaccination of birds. Several approaches have been used to attenuate *Eimeria* including serial passage in embyonated eggs (Gore *et al*. 1983) and generation of precocious strains that undergo fewer cycles of asexual reproduction (Jeffers, 1975; Shirley *et al*. 1995). Another approach has been to expose *Eimeria* oocysts to gamma irradiation which does not affect sporozoite invasion but does prevent asexual parasite development in the host (Jenkins, Chute and Danforth, 1997).

Passage of *Eimeria* through eggs rendered them less pathogenic, although some species were not found to be amenable to this process (Shirley and Long, 1990). The vaccine, Livacox® is an example of an embryo-adapted *E. tenella* line. Precociousness refers to a naturally occurring population of parasites that complete their lifecycle from sporozoite to oocyst 20–30 h faster than their fellow parasites from the same parent. This is a selectable trait and sometimes this is accompanied with a decrease in proliferative capacity and pathogenicity (Jeffers, 1975). Paracox® was the first line of vaccines that utilized this feature for live vaccines with a better safety profile (Williams, 2002). Several newly developed precocious vaccines have been reported in different parts of the world (Li *et al*. 2004; Vermeulen, 2004; Kawazoe *et al*. 2005), where it was observed that selection for precociousness did not always result in a reduction in pathogenicity (Kawazoe *et al*. 2005).

Vaccine strains selected for naturally occurring low pathogenicity were included in NobilisCox ATM® (Vermeulen, Schaap and Schetters, 2001). Where most vaccines are claimed to contain drug-sensitive strains, the latter vaccine comprises strains with a defined tolerance for specific drugs. Thereby it allows the concomitant use of certain ionophores until immunity is fully developed. The vaccine strains are, however, fully susceptible to drugs such as diclazuril and toltrazuril, which allows removal if preferred. Recently Li *et al*. (2004), selected ionophore-tolerant precocious strains for a similar purpose and Kawazoe *et al*. (2005), demonstrated that a degree of ionophore tolerance is a natural feature of different *Eimeria* strains that had not had previous contact with these drugs.

Problems with antigenic variability between different *Eimeria* species and strains becomes more and more evident, especially when live *E. maxima* vaccine strains are applied to induce protective immunity (Smith *et al*. 2002). Only Paracox® and NobilisCox ATM® have included antigenically different strains where the two strains in the latter vaccine appear to act synergistically (Vermeulen, 2004). Paracox® contains oocysts of eight precocious lines from seven different species of *Eimeria*.

Efficacy of vaccination was often associated with difficulty in the application of the vaccine. However, with improved administration of live oocysts, chickens develop immunity more readily as a consequence of early cycling of the parasites (Vermeulen, Schaap and Schetters, 2001; Williams, 2002). Novel methods of vaccine delivery such as spray-on day-old birds is widely seen as the best method to trickle the infection in the chicks. *In ovo* application of live coccidial vaccines (reviewed in Shirley, Smith and Tomley, 2005), such as Inovocox®, Embrex, may also turn out to be an effective route of delivery as it induces early immunity, reduced bird stress and results in a more precise and uniform dosing system. Now that increasing numbers of similar vaccines become commercially available, objective criteria need to be set for these products as presently no monograph exists regarding safety and efficacy requirements.

Recombinant vaccines: A major drawback of live vaccines is their limited shelf-life and the relatively high production costs, especially when attenuated vaccines are to be produced. It should be realised in this respect that live vaccinal oocysts are to be produced by chickens and these should provide enough vaccine material to inoculate forty billion broilers each year. Thus there is a great need for mass

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production and mass application of effective vaccine and many groups are interested in examining the potential of using recombinant antigen vaccines (Jenkins, 1988, 2001; Vermeulen, 1998). Recombinant vaccines may provide the best long-term solution, in particular as more drugs will be banned and legislative requirements for live vaccines will become more strict.

Selection of relevant antigens is a crucial step in developing effective sub-unit vaccines and work has focused on identifying antigens/proteins essential for parasite survival in the host or that are recognized by protective antibodies or T cells. Microneme proteins are common to apicomplexan parasites and are involved in host cell adhesion and penetration making them promising targets in developing recombinant vaccines. Several microneme genes of *E. tenella* have been identified and cloned (Ryan, Shirley and Tomley, 2000) and the EtMIC2 protein has been localised at the point of parasite entry to the host cell, later dispersing over the surface of the infected cell (Tomley *et al.* 1996). The antigens, EtMIC2 and EtMIC4 have shown some protective effect (Dalloul and Lillehoj, 2005; Du and Wang, 2005), and a recent study showed that *in ovo* vaccination with EtMIC2 gene stimulated intestinal protective immunity against *E. tenella* and *E. acervulina* (Ding *et al.* 2005). Further work showed that administration of short oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODNs) which are known to enhance both innate and adaptive immune responses (Krieg, 1995), along with the MIC2 antigen *in ovo* enhanced the immunogenicity of the vaccine preparation (Dalloul *et al.* 2005). A major challenge in developing an effective recombinant vaccine is to ensure optimal delivery strategies to process and present the antigens in an appropriate manner to the immune system (Jenkins, 2001). In this regard, studies examining delivery of antigens using live viral or bacterial vectors may offer a solution as these vectors are able to stimulate cell mediated immune responses (Kim, Jenkins and Lillehoj, 1989; Cronenberg *et al.* 1999). Immunogenic soluble/cytoplasmic proteins such as LDH (Vermeulen, 2004) and enzymes from the anti-oxidant pool (SOD and 1Cys-peroxidoxin) have been shown to be promising candidates, since they are recognized by CD4+ and CD8+ T cells, induce production of IFN-γ and partial protection was evoked in vaccinated chickens using *Salmonella typhimurium* expressed genes (Kuiper *et al.* 2001).

Increasing knowledge of the action of immune cytokines may make these useful adjuvant reagents. Enhancement of the host immune response to vaccination with MIC2 antigen was achieved by co-injection of the chicken IL-2 gene (Ding *et al.* 2005). Song *et al.* (2000) and Min *et al.* (2001), used pcDNA3-1E plasmid and co-injected that with cytokine genes into 1 day-old chicks, although the protection achieved in this study was minimal (<30% oocyst reduction). Wu *et al.* (2004) found the Et1A and TA4 genes were effective vaccine candidates resulting in improved weight gain and >60% reduced oocyst output. DNA plasmid deposition was applied using *Salmonella typhimurium* bacteria, pcDNA5401, resulting in a 50% oocyst reduction following challenge (Du and Wang, 2005).

The new approaches involving the use of viral vector delivery systems may be the most promising way forward in the challenge to produce, sustainable cost-effective *Eimeria* vaccines suitable for mass application. Fowlpox and Herpes virus of turkeys are promising candidates as they are able to harbour the insert sizes needed to express the multiple *Eimeria* genes necessary to control the various species of parasite (Cronenberg *et al.* 1999; Boyle and Heine, 1993). These live delivery systems will allow appropriate processing and presentation of antigens to the immune system in conjunction with MHC molecules to stimulate protective cell-mediated immune responses.

Eimeria – *concluding remarks*

The major challenges facing control of poultry coccidioses are that resistance is developing to nearly all of the existing in-feed anti-coccidial drugs, more anti-coccidial drugs are being banned from use in food animals and there are very few new drugs being developed. Current vaccines are costly to produce, strain- and species-specific immunity means that a cocktail of antigens may be required to give adequate protective immunity and there are welfare issues that can no longer be overcome in using millions of chickens to produce the live vaccines to the extent needed. Recombinant vaccines may be the long-term sustainable solution and the major challenge ahead is to devise effective ways to deliver these antigens to the immune system in order to stimulate appropriate protective immunity.

TOXOPLASMA

Toxoplasma gondii is one of the most successful parasites worldwide, capable of infecting all warm blooded animals and it is currently estimated that a quarter of the world's population are infected. In general, infection with *T. gondii* results in mild clinical symptoms with the parasite persisting for the lifetime of the host. However, with such a wide spectrum of different hosts, there are exceptions to this generalised view of the host-parasite relationship both between and within different host species (Innes, 1997). In human infection, pregnant women and immuno-compromised individuals are the main risk groups although ocular disease and psychiatric

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disorders may also result from infection of immunocompetent individuals (McAllister, 2005). The parasite is also a major cause of abortion in sheep and goats in particular in the the more temperate regions in the world such as New Zealand, France, UK and Norway where climatic conditions for oocyst survival and sporulation are optimal (Lind and Buxton, 2000). Congenital infection is also a problem in farmed pigs (Dubey and Urban, 1990). Food animals such as pigs, sheep and goats may harbour tissue cysts of *T. gondii* which may be transmitted to people through consumption of undercooked meat (Tenter, Heckeroth and Weiss, 2000). Cats are the definitive host of the parasite and following a primary infection with *T. gondii* a sexual cycle takes place in the epithelial cells of the gut resulting in the production of oocysts which are shed in the faeces. Control strategies against the parasite involve education concerning transmission routes to avoid becoming infected, in particular for pregnant women. Drug treatment with spiramycin (Desmonts and Couvreur, 1979), pyrimethamine and sulfadiazine have been used in cases of congenital infection in pregnant women and in treatment of congenitally infected children to alleviate the development of retinochoroiditis later in life (Roberts and McLeod, 1999). Treatment to help prevent reactivation of toxoplasma infection in HIV patients has involved the drugs, trimethoprim-sulfamethoxazole and dapsone-pyrimethamine or fansidar (Bozzette *et al*. 1995, Podzamczer *et al*. 1995). Although these drugs show some activity against the actively multiplying tachyzoite stage of the parasite there are no effective drugs available that are able to act against the tissue cyst stage of the parasite and hence cure people or animals of a persistent infection (Huskinson-Mark, Araujo and Remington, 1991). There are also concerns in using drugs to treat pregnant women due to the potential toxicity or teratogenic effect on the developing foetus (Derouin, 2000). In most immuno-competent individuals, infection with *T. gondii* results in the development of a protective immune response against further disease; therefore a control strategy based on vaccination would be an additional option to combat the parasite. We will discuss the main transmission routes of infection, risk groups and targets for vaccination along with current and future immunisation strategies.

Transmission routes

The main transmission routes of *T. gondii* to people and animals has recently been reviewed by Tenter *et al*. (2000). Cats are the definitive host of the parasite and young animals usually become infected for the first time by eating the tissue cyst stage of the parasite (Fig. 1) contained in infected rodents and birds. Parasites then invade the enteroepithelial cells where the sexual cycle takes place resulting in the formation of oocysts which are shed in the faeces for 5–14 days post-infection (Dubey and Lindsay, 1996). Oocysts undergo sporogony outside the host resulting in the formation of two sporocysts, each containing four sporozoites. This infective stage of the parasite is very resistant in the environment and can remain infective for up to 18 months depending on climactic conditions. Heating of oocysts to 70 °C for 2 minutes will render them uninfective, but they are resistant to common disinfectants such as bleach (Frenkel, 2000). Intermediate hosts may acquire infection through the consumption of sporulated oocysts present in contaminated food and drink. Studies examining California sea otters found that toxoplasmosis was a major cause of mortality in this species due presumably to contamination of the oceans by oocysts (Kreuder *et al*. 2003).

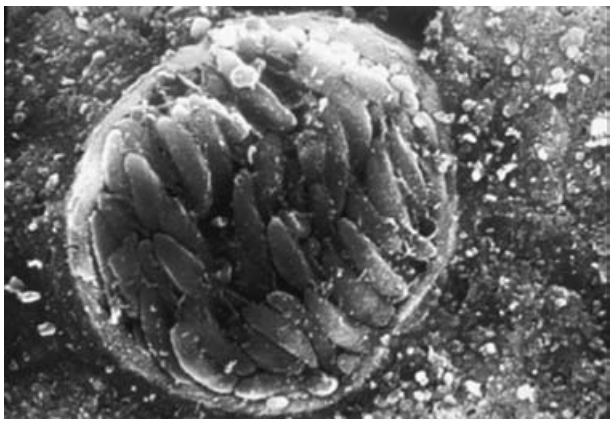

Fig. 1. Scanning electron micrograph through a *Toxoplasma gondii* tissue cyst within brain tissue. Courtesy of Professor David J. P. Ferguson, Department of Pathology, University of Oxford.

Oocysts excyst in the gut and the sporozoites invade and multiply within the gut cells and mesenteric lymph node cells before initiating a parasitaemia and spreading to other parts of the body (Buxton, 1998). Asexual multiplication by tachyzoites takes place within many different types of nucleated host cell where parasites divide by a process of endodyogeny within the parasitophorous vacuole (Lingelbach and Joiner, 1998). Tachyzoites eventually burst out of the cell going on to invade and multiply within other cells. Counter-pressure by the host immune system is thought to trigger differentiation of the parasite into the slow replicating bradyzoite stage which persist and divide further within the host inside tissue cysts (Frenkel, 2000). Tissues containing bradyzoite cysts are also infectious for intermediate hosts and it is recommended that meat should be cooked to a minimum temperature of 67 °C or frozen at −13 °C or lower to kill parasite tissue cysts (Hill and Dubey, 2002). Studies in livestock have indicated that meat from pigs, sheep and goats poses the highest risk for infection, followed by free-range poultry and game animals, then meat from cattle or

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buffalo which is considered to be a comparatively lower risk. Thus people and animals become mainly infected via consumption of oocysts or bradyzoites within tissue cysts. Consumption of milk from a recently infected mother may transmit the infection and the parasite may also be passed vertically from mother to foetus during pregnancy. Finally, humans may become infected through receiving an organ transplant from a *T. gondii* infected donor especially since this is accompanied by immune-suppressive treatment (Dubey and Beattie, 1988).

Risk groups

First of all, pregnancy itself is an important risk factor for toxoplasmosis (Avelino *et al*. 2004). If a pregnant woman becomes infected for the first time during pregnancy there is a risk that she may transmit the infection to the foetus. The risk of transmission to the foetus increases throughout gestation although the disease severity and consequences to the foetus decrease as pregnancy progresses (Remington and Desmonts, 1990). Consequences of foetal infection may be death or clinical symptoms at birth including retinochoroiditis, intracranial calcifications and hydrocephalus. Congenital infection is also a major cause of abortion and neonatal mortality in farmed sheep and goats (Buxton, 1998). Toxoplasmosis may also be a life-threatening illness in immuno-compromised individuals where it may present as a reactivation of a previously acquired infection. Toxoplasmosis has been cited as the third most frequently diagnosed cause of food related deaths in the USA (Mead *et al*. 1999). *Toxoplasma* encephalitis is a major cause of death in AIDS patients (Ammassari *et al*. 1996) although the new anti-retroviral therapies are helping to combat this by supporting the patient's immune system. Patients undergoing immunosuppressive therapy for cancer treatment or organ transplants may also be at risk from *Toxoplasma* infection. Recent studies have highlighted the development of ocular disease in immunocompetent individuals who have acquired the disease postnatally (Holland, 1999). In addition, there have been reports of a link between toxoplasmosis and psychiatric disorders such as schizophrenia (McAllister, 2005), which may provoke a re-evaluation of the public health risk posed by *T. gondii*. Several animal species such as marsupials and new world monkeys are very vulnerable to infection with the parasite which often results in fatal disease (Innes, 1997). In addition, *Toxoplasma* encephalitis was found to be a major cause of death in wild Californian sea otters (Kreuder *et al*. 2003).

In most immuno-competent animals infection with *T. gondii* results in development of protective immunity against disease. There is also little evidence that strain-specific immunity is a problem with this parasite (Smith and Frenkel, 2003). Therefore control of disease by vaccination has a high likelihood of success. There is a commercial vaccine available to protect against *Toxoplasma* abortion in sheep and goats, Ovilis® Toxovax (Intervet).

Targets for vaccination

Linked to what we currently understand concerning the main transmission routes and disease manifestations, targets for a vaccination strategy would include: (1) Vaccination to limit acute parasitaemia and to protect against congenital toxoplasmosis; (2) Vaccination to reduce tissue cysts; and (3) Vaccination to reduce oocyst shedding in cats to limit environmental contamination. Before discussing current approaches to vaccination we will briefly summarise protective immune responses as this is relevant to the rational design of novel vaccine strategies.

Protective immune responses

Stimulation of the innate immune system occurs early in *T. gondii* infection as parasites are able to stimulate macrophages directly resulting in the production of IL-12 which in turn can stimulate NK cells to produce IFNγ (Gazzinelli *et al*. 1993). This early induction of IFNγ may be important in inhibiting tachyzoite proliferation during the early stages of infection and will also provide the appropriate cytokine environment during the priming of the adaptive immune response resulting in a bias towards a Th1-type pro-inflammatory immune response (Gazzinelli *et al*. 1996). The regulatory cytokine IL-10 is important to help protect against the potential immunopathology caused by a vigourous Th1-type immune response (Gazzinelli *et al*. 1996). As *T. gondii* is an obligate intracellular parasite, cell mediated immune mechanisms are thought to be important in controlling a primary infection whereas, antibody may be more important during secondary challenge. Much of our understanding of immune responses induced following *T. gondii* infection has been determined using mouse models. Adoptive transfer experiments have emphasised the importance of T cells in protective immunity, in particular CD8+ T cells (Parker, Roberts and Alexander, 1991; Khan, Ely and Kasper, 1994). Transfer of protective immunity was also achieved using gut intraepithelial lymphocytes from immune mice emphasising the importance of mucosal immunity as this is the main site of entry to the host for the parasite (Buzoni-Gatel *et al*. 1997). In addition, a key cytokine involved in protective immunity is IFNγ (Scharton-Kersten *et al*. 1996). While CD8+ T cells have been shown to be cytotoxic for parasite infected cells (Hakim *et al*. 1991; Subauste, Koniaris and Remington, 1991) they may also exert their

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anti-parasite effect through production of IFNγ (Suzuki and Remington, 1990). CD4+ T cells and NK cells are also known to produce IFNγ and studies have shown that both CD4+ and CD8+ T cells are required for development of protective immunity (Suzuki and Remington, 1988; Gazzinelli *et al*. 1991; Curiel *et al*. 1993). The importance of T cells and IFNγ in protective immunity in human infection is illustrated by recrudescence of a previous *T. gondii* infection in HIV patients where the T cell response is impaired (Luft *et al*. 1984). Other studies in humans have shown that both CD4+ and CD8+ T cells are cytotoxic for parasite-infected cells (Montoya *et al*. 1996). Using a lymphatic cannulation model to monitor the *in vivo* kinetics of a primary *T. gondii* infection in sheep it was shown that the first lymphoblasts produced responding to the infection were CD4+, with a switch to CD8+ becoming the predominant lymphoblast population coinciding with control of the parasite (Innes and Wastling, 1995). While antibody responses are not thought to be so important during a primary infection with *T. gondii* they are likely to play a more significant role during a secondary challenge. Passive transfer of immune serum or monoclonal antibodies affords some protection following challenge (Krahenbuhl, Gaines and Remington, 1972; Johnson, MacDonald and Neoh, 1983) and B cell-deficient mice had an impaired resistance to the parasite (Kang, Remington and Suzuki, 2000).

Taken together, these studies emphasise the importance of a Th 1-type immune response involving CD4+, CD8+ and IFNγ as the main mediators of protective immunity against a primary infection with *T. gondii*. However, in attempting to induce these responses using various vaccine preparations one needs to be aware of the role of regulatory cytokines in balancing and controlling the potential immunopathology caused by a vigorous inflammatory response. The other main challenge is to devise methods to enable processing and presentation of vaccine antigens within the correct MHC background to stimulate appropriate T cell responses. This is perhaps why most successful vaccines so far against *T. gondii* infection have comprised live organisms that undergo limited multiplication within host cells and thus present antigens to the immune system in the correct MHC context.

Vaccination strategies against T. gondii

Vaccination to limit acute parasitaemia and to protect against congenital toxoplasmosis: This type of vaccine would protect against foetal disease in pregnant women and farm livestock and in addition may provide protection against acquired toxoplasmosis leading to development of ocular disease and potential psychiatric disorders due to *T. gondii* infection and persistence in hosts.

The only commercially available vaccine against *T. gondii* comprises live attenuated tachyzoites of the S48 strain (O'Connell, Wilkins and Te Punga, 1988; Wilkins, O'Connell and Te Punga, 1988) that affords protection against *Toxoplasma*-induced abortion in sheep (Buxton *et al*. 1993). The S48 strain was originally isolated from an aborted lamb in New Zealand, has been passaged over 3000 times in mice and has lost the ability to form tissue cysts or oocysts. The tachyzoites undergo limited multiplication in the host and are able to induce appropriate cell-mediated immune responses (Innes *et al*. 1995*a*, *b*). Immunity induced after vaccination protected against abortion following challenge with oocysts and this immunity was long lasting with sheep still immune to a challenge administered 18 months after the initial vaccination (Buxton *et al*. 1993). The vaccine is administered prior to mating and is effective after a single shot. As the vaccine is live it does have a relatively short shelf-life and care should be taken with administration. This highly effective vaccine is licensed for veterinary use only and, because no data are available, it is considered not safe enough to use in people. Therefore, studies towards developing a human vaccine to prevent congenital toxoplasmosis have focused on killed vaccines using defined immuno-dominant antigens and different delivery strategies.

The SAG 1 molecule is an immunodominant surface protein found on tachyzoites and is one of the most extensively studied antigens as it is able of inducing both T cell and antibody responses (Khan, Smith and Kaspar, 1988; Mineo *et al*. 1993). While the choice of antigen is an important factor, what may be more important is the choice of adjuvant or delivery system to enable effective processing and presentation of the antigen to the immune system. Various strategies have been tried with some success including incorporation of antigen into liposomes (Bulow and Boothroyd, 1991; Roberts, Brewer and Alexander, 1994) or administration with cholera toxin as an adjuvant (Debard, Buzoni-Gatel and Bout, 1996). Immunostimulating complexes (ISCOMS) have been used as an adjuvant as they are known to stimulate both antibody and cell mediated immune responses and showed protection when used as an adjuvant with *T. gondii* to immunise mice (Uggla *et al*. 1988), although they were not able to induce sufficient immunity to protect against abortion in sheep (Buxton *et al*. 1989). Recent studies have examined the use of DNA vaccination as this approach is known to be effective in inducing MHC class I restricted CD8+ T cell responses that we know are protective against *T. gondii* infection. Genetic vaccination with a cDNA encoding SAG1 protected mice against a lethal challenge with tissue cysts of ME49 strain Toxoplasma (Angus *et al*. 2000). DNA vaccination with SAG 1 also protected against acquired *T. gondii* infection in mice, but did

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not protect against congenital infection (Couper *et al*. 2003). Further studies showed that mice immunised with plasmids expressing both SAG1 antigens and a dense granule antigen GRA4, which is expressed by both tachyzoites and bradyzoites, along with a plasmid encoding GM-CSF were able to induce protection against acute and persistent *T. gondii* infection and partial protection against congenital toxoplasmosis (Mevelec *et al*. 2005). This study emphasises the importance of using a multivalent vaccine combining antigens from different life-cycle stages of the parasite and using selected immune cytokines as adjuvants to customise an appropriate immune response. Studies using recombinant vaccinia virus constructs with selected genes of *T. gondii* have also shown promising results in inducing protective immunity (Roque-Resendiz, Rosales and Herion, 2004) and this approach, like the use of DNA vaccination, has the advantages of being able to introduce several parasite antigens within the vaccine and induce appropriate protective cell mediated immune responses.

While these results appear very promising using mouse models it is difficult to extrapolate the findings to predict the outcome of using such a vaccination approach in people. Rats may be a more relevant model of human congenital toxoplasmosis as rats would pass the parasite vertically when given a primary challenge during pregnancy and immunisation of rats prior to pregnancy would protect against maternofoetal transmission during pregnancy (Zenner *et al*. 1993). Pregnant sheep may offer a relevant intermediate animal model to test out a vaccine to prevent congenital infection as the disease in pregnant sheep and pregnant women is very similar. In addition, the induction of mucosal immunity through oral or intranasal administration of vaccine candidates and adjuvants (Bourguin, Chardes and Bout, 1993; Debard *et al*. 1996; Stanley *et al*. 2004) may provide an effective route of administration for human vaccination as the natural site of entry to the host is through the intestinal tract.

Vaccination to reduce tissue cysts: A vaccine to prevent tissue cyst formation in food animals would be highly desirable as this would help reduce transmission to people through the consumption of under-cooked infected meat. In addition, such a vaccine may be important in people to reduce the number of persistently infected individuals who may be at risk from developing disease due to immune dysfunction later in life. A reduction in the numbers of tissue cysts in pigs was achieved by immunisation with the RH strain of *T. gondii*, which like the S48 strain only undergoes limited multiplication in the host (Dubey, Urban and Davis, 1991). The protective effect of immunisation using RH tachyzoites was improved by using oligodeoxynucleotides containing immunostimulatory CpG motifs (CpG ODN), which are known to enhance Th1-type immune responses, as an adjuvant (Kringel *et al*. 2004). Over half of the pigs vaccinated with RH strain *T. gondii* and CpG ODN had no demonstrable tissue cysts following challenge with oocysts (Kringel *et al*. 2004). However, only partial protection against tissue cysts was observed in pigs immunised using a crude fractionation of *T. gondii* rhoptry proteins incorporated into ISCOMS (Garcia *et al*. 2005). The authors discussed the improvement of their vaccine by incorporating a cocktail of different antigens from different life cycle stages as studies in mice have shown that immunisation with plasmids encoding GRA1, GRA7 and ROP2 gave some protection against a lethal challenge with *T. gondii* tissue cysts and the number of brain tissue cysts observed was significantly lower than in control animals (Vercammen *et al*. 2000). The importance of using cyst-specific antigens to protect against development of tissue cysts was discussed by Alexander *et al*. (1996). An immunodominant antigen expressed in tissue cysts containing bradyzoites is MAG1 (Parmley *et al*. 1994). Immunisation of mice with recombinant MAG1 led to a significant reduction in cerebral tissue cysts following challenge (Parmley, Slifer and Araujo, 2002). An antigenic cocktail of distinct microneme antigens, MIC2, MIC3, MIC4, M2AP and AMA1 was recognised by antibodies and T cells from individuals with acquired and congenital toxoplasmosis (Beghetto *et al*. 2005). DNA immunisation with these antigens led to an 84% reduction in brain tissue cyst burden in mice following challenge (Beghetto *et al*. 2005). These studies emphasise the importance of selecting appropriate stage–specific antigens to achieve the desired biological effect. Targetting bradyzoite antigens in a vaccine preparation may also be beneficial as this is the stage that is first encountered by the immune system if transmission occurs through the consumption of tissue cysts within infected meat.

Vaccination to reduce oocyst shedding in cats: Contamination of the environment by oocysts is a major source of infection for intermediate hosts (Tenter *et al*. 2000) and recent large-scale outbreaks of clinical toxoplasmosis in Canada and Brazil were associated with oocyst contamination of water supplies (Bowie *et al*. 1997; Bahia-Oliveira *et al*. 2003). Infection of a variety of marine mammals (Dubey *et al*. 2003) has highlighted the extent of oocyst contamination of oceans. Therefore a key strategy in controlling *T. gondii* infection would be to reduce the environmental contamination with oocysts which would involve managing the infection in cats.

A mutant strain of *T. gondii*, T-263, was developed that would only undergo partial development in the gut of the cat and therefore did not result in the

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production of oocysts (Frenkel *et al*. 1991). Oral immunisation of cats with T-263 bradyzoites, resulted in 84% of cats not shedding oocysts following challenge (Frenkel *et al*. 1991). This protection was improved by administering two doses of the live vaccine (Frenkel *et al*. 1991; Freyre *et al*. 1993). A large-scale trial of the vaccine was conducted over a three year period on eight commercial pig farms in the USA. Young cats were trapped and vaccinated with the T-263 vaccine and the overall result was a decrease in environmental contamination with oocysts resulting in decreased seroprevalence of other intermediate hosts including the farmed pigs (Mateus-Pinilla *et al*. 1999). Further work using a deterministic dynamic computer simulation model to evaluate the T-263 vaccine showed that the decrease in seroprevalence in the pigs was related to number of cats on the farm, oocyst survival and vaccination of cats (Mateus-Pinilla, Hannon and Weigel, 2002). Although the vaccine has proved to be efficacious in trials, it is produced *in vivo* in mouse brains which is a major drawback in terms of large-scale production and cost. This vaccine is a live vaccine and is administered orally. The vaccine is kept frozen until delivery to maintain viability of bradyzoites (Choromanski *et al*. 1995). Additional studies looking at vaccination of cats has used a recombinant feline herpesvirus type 1 (FHV1) vector expressing ROP2 antigen of *T. gondii* which resulted in reduced numbers of cerebral parasites (Mishima *et al*. 2002).

Toxoplasma – *concluding remarks*

Toxoplasma is a fascinating and ubiquitous parasite with numerous intermediate hosts and the cat family as the only definitive host. The clinical manifestation of disease in different hosts along with knowledge of the importance of the various transmission routes means that different control and vaccination strategies may need to be applied to tackle this parasite. Points in favour of vaccination as a method of disease control are that following a primary infection with the parasite the host generally develops an effective protective immunity against disease and, unlike the situation with *Eimeria* parasites, there is good cross-protection among different strains of *T. gondii*. However, protective immunity involves stimulation of cell mediated immune responses, in particular CD8+ T cells and IFNγ which requires endogenous processing and presentation of antigens in association with MHC class 1 antigens. This perhaps explains the comparative success of using live vaccines compared to killed antigen preparations. However considerable progress has been made in identifying relevant antigens of the different asexual life cycle stages of *T. gondii* and those that are involved in cell entry and intracellular survival of the parasite. Improved knowledge of pro-inflammatory and regulatory cytokine networks and antigen delivery strategies, involving adjuvants, live virus vectors and DNA vaccination has greatly progressed our understanding of how to induce and regulate protective immune responses. As a result of these studies there is real optimism in developing cost-effective new vaccines that may be suitable for large-scale production. Effective vaccines to prevent oocyst shedding by cats and tissue cyst formation in food animals would have great impact on environmental contamination and consequently for public health, although there may be interesting consequences for herd immunity if infection rates dropped. Control strategies to prevent congenital transmission and acute infection with the parasite would be of considerable benefit due to the costs incurred in managing individuals with mental disabilities, ocular disease and perhaps even psychiatric illness. Clearly a vaccine strategy for use in people will have to overcome more stringent safety requirements than those used for animals and it is unlikely that a live vaccine would be considered suitable. A further consideration for vaccination to prevent congenital disease is that natural changes in immune cytokine regulation during pregnancy result in the cytokine environment at the maternal-foetal interface being predominantly Th2-type and induction of a Th1-type response may be dangerous for the pregnancy. Therefore, as it is important to induce a Th1-type immune response to protect against *T. gondii* it would be advisable to administer a vaccine prior to pregnancy.

NEOSPORA

Neospora parasites were first recognised as causing disease in dogs (Bjerkas, Mohn and Presthus, 1984) and were isolated into tissue culture and identified as a new genus with *Neospora caninum* as the type species in 1988 (Dubey *et al*. 1988*a*, *b*). Since then the parasite has also been found in cattle, goats, deer, horses and sheep with antibodies being found in water buffalo, coyotes, red foxes and camels (Dubey, 1999). A separate species, *N. hughesi*, has been suggested for the parasite in horses based on morphological and molecular differences (Marsh *et al*. 1998). The definitive host of the parasite is the dog (McAllister *et al*. 1998; Basso *et al*. 2001) and coyotes have also been found to shed oocysts (Gondim *et al*. 2004*a*). Although *N. caninum* is closely related to *T. gondii* morphologically, genetically and antigenically (Dubey and Lindsay, 1996; Tenter and Johnson, 1997; Howe *et al*. 1998) there are differences between them in their biology, host-parasite relationship and disease profiles. *N. caninum* appears to have a more limited intermediate host range than *T. gondii* (Dubey, 1999) and there is no good evidence that *N. caninum* infects and causes disease in humans (Graham *et al*. 1999). The main clinical

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manifestations of infection by *N. caninum* are seen in dogs and cattle. Interestingly, *T. gondii* is not thought to be a significant cause of disease in cattle (Dubey, 1986; Esteban-Redondo and Innes, 1997) whereas *N. caninum* is emerging as a major cause of reproductive failure in cattle worldwide (Dubey, 2003). There are currently very few effective control strategies against bovine neosporosis. New information concerning the life cycle and transmission routes of the parasite has been helpful in educating farmers on how to minimise exposure of cattle to infective stages of *N. caninum* and although some progress has been made in assessing susceptibility of parasites to chemotherapeutic agents (Lindsay *et al.* 1996; Gottstein *et al.* 2001) and in treatment of canine neosporosis with sulfonamides, pyrimethamine and clindamycin (Barber and Trees, 1996), there are no drugs that will cure animals of the parasite.

An interesting feature of *N. caninum* infection in cattle is that vertical transmission of the parasite is highly efficient (Pare, Thurmond and Hietala, 1996; Davison, Otter and Trees, 1999) and this may occur over several generations and in successive pregnancies (Barr *et al.* 1993; Bjorkman *et al.* 1996; Wouda, Moen and Schukken, 1998) suggesting that cattle do not develop very good immunity against vertical transmission of the parasite (Innes *et al.* 2002). It is unknown whether this is mainly due to recrudescence of an endogenous infection or due to a new infection (Innes *et al.* 2002). Cattle which have experienced an abortion due to neosporosis have a significantly decreased chance of having a repeat abortion due to the same infectious agent (Anderson *et al.* 1995; Wouda *et al.* 1998), implying that cattle can develop a degree of protective immunity against abortion. Further evidence for this came from an investigation of a point source outbreak showing that those cattle which had evidence of prior exposure to *N. caninum* were less likely to abort compared with those undergoing a primary infection (McAllister *et al.* 2000). Additional evidence from laboratory studies has shown that experimental infection of naïve animals prior to mating afforded immunity against both abortion and vertical transmission of the parasite following challenge during pregnancy (Liddell *et al.* 1999; Innes, *et al.* 2001; Buxton *et al.* 2001). In addition, persistently infected cattle were protected against a challenge that induced foetopathy in naïve control animals (Williams *et al.* 2003). Therefore a vaccination strategy to control the disease may be possible and the prevention or reduction of abortion may be a more feasible goal than to try and prevent vertical transmission. We will focus our discussion on the disease in cattle looking at the transmission routes, host immune responses, host-parasite relationship and current strategies to develop effective vaccines against bovine neosporosis.

The disease in cattle, parasite transmission and life cycle

Epidemiological studies in several countries have shown that cattle infected with *Neospora caninum* are three to seven times more likely to have an abortion compared with uninfected cattle, with the highest risk during a first pregnancy (Thurmond and Hietala, 1997*a*; Moen *et al.* 1998; Wouda *et al.* 1998). Adult cattle rarely show clinical symptoms following infection and disease manifests in the placenta and developing foetus (Innes *et al.* 2002; Buxton, McAllister and Dubey, 2002). Clinical consequences of infection include abortion of the foetus, birth of a weak calf sometimes showing neurological symptoms or birth of a clinically normal but persistently infected calf (Dubey and Lindsay, 1996). The clinical outcome is likely to be related to the timing of infection during pregnancy (Innes *et al.* 2002). Evidence from experimental studies indicates that infection occurring early in gestation has more severe consequences for the foetus than infections occurring later in gestation (Barr *et al.* 1994; Buxton *et al.* 1998; Williams *et al.* 2000; Maley *et al.* 2003; Macaldowie *et al.* 2004). Economic losses associated with the disease include costs associated with loss of calf, fertility problems and increased calving interval, reduced milk production, reduced value of stock and increased likelihood of culling (Thurmond and Hietala, 1997*b*; Trees *et al.* 1999; Dubey, 2003).

Neospora caninum may be transmitted to cattle via consumption of feed or water contaminated with the oocyst stage of the parasite or by vertical transmission of the tachyzoite stage from dam to foetus during pregnancy (Dubey, 2003). Dogs have recently been identified as a definitive host of the parasite (McAllister *et al.* 1998; Basso *et al.* 2001). Oocysts may be shed in the faeces of acutely infected dogs that acquire the infection through the consumption of infected bovine placentas (Dijkstra *et al.* 2001) or other bovine tissues (Gondim, Gao and McAllister, 2002). The oocyst stage of the parasite is thought to persist in the environment but currently little is known about the environmental conditions that may favour oocyst survival or the frequency of oocyst shedding by dogs (Dubey, 2003). Following ingestion of oocysts, the parasites excyst in the gut and invade and multiply within host cells. The tachyzoite stage of the parasite actively invades host cells and multiplies by a process called endodyogeny resulting in many tachyzoites which burst from the cell ready to invade new cells and resume rapid multiplication (Dubey and Lindsay, 1996; Hemphill, 1999). Using this process the parasite can disseminate via the circulation throughout the host (Okeoma *et al.* 2004). The parasite can only multiply within host cells and it is thought that under pressure from the immune response of the host, the parasite differentiates

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into the slower multiplying bradyzoite stage. Bradyzoites are usually observed within tissue cysts in neural tissues (brain and spinal cord) and this is thought to be how the parasite may cause persistent infection in cattle (Dubey and Lindsay, 1996). Vertical transmission from dam to foetus may occur following an exogenous challenge during pregnancy or may result following recrudescence of an existing persistent infection. A characteristic of bovine neosporosis is the high rate of vertical transmission estimated at between 78–95% (Pare *et al*. 1996; Davison *et al*. 1999) and, as discussed previously, vertical transmission can occur over several generations and in consecutive pregnancies (Bjorkman *et al*. 1996).

Host immune responses

In any host-parasite relationship a vast array of different immune responses are induced against the various life cycle stages of the parasite. Some of these immune responses will be protective to the host, others protective to the parasite, some may cause pathology in the host and others may be largely irrelevant. In the following sections we will discuss the different roles of the host immune response and how this contributes to our understanding of the host-parasite relationship, disease pathogenesis and immunological strategies to control the disease.

The tachyzoite stage of *N. caninum* actively invades and multiplies within various cells of the host (Hemphill, 1999) and the intracellular location of the parasite suggests that cell mediated immune responses are likely to play a significant role in protective immunity (Marks *et al*. 1998). Interferon gamma (IFNγ) and tumour necrosis factor alpha (TNFα) are known to inhibit intracellular multiplication of *N. caninum* significantly (Innes *et al*. 1995*c*; Yamane *et al*. 2000). The cytokines IFNγ and interleukin 12 (IL-12) were shown to be important components of protective immunity using mouse models of infection (Khan *et al*. 1997; Baszler *et al*. 1999) and IFNγ knockout mice showed a significantly increased vulnerability to *N. caninum* infection (Dubey *et al*. 1998). The importance of CD4+ T cells in protective immunity was highlighted in a study where mice were treated *in vivo* with antibodies to deplete CD4+ or CD8+ T cells prior to challenge with *N. caninum* (Tanaka *et al*. 2000). In the group of mice where CD4+ T cells were depleted, all mice died within 30 days of the challenge; in contrast, no mice died within this time period in the control group or the group where CD8+ T cells had been depleted (Tanaka *et al*. 2000). Supporting a prominent role for CD4+ T cells in protective immunity were studies showing that *N. caninum*-specific CD4+ T cells, from infected cattle, were able to lyse parasite-infected autologous target cells directly *in vitro* (Staska *et al*. 2003). In addition, recent work has also shown that bovine NK cells are able to kill *N. caninum*-infected cells (Boysen *et al*. 2006). Evidence for the importance of antibody responses came from studies using μMT knock out mice as these were found to be significantly more susceptible to infection than the wild type mice (Eperon *et al*. 1999).

While we still know comparatively little concerning induction, function and regulation of protective immune mechanisms against *N. caninum* parasites in cattle, current data would support an important role for CD4+ T cells and pro-inflammatory cytokines such as IFNγ.

Changes to the host-parasite relationship during pregnancy

Neosporosis is a disease that manifests during pregnancy where the developing foetus is particularly vulnerable. Various changes occur in the maternal immune response to enable the dam to support the pregnancy and prevent immunological rejection of the semi-allogeneic foetus (Raghupathy, 1997). These natural changes in the immune system may favour the parasite and help to explain disease pathogenesis in pregnancy. Relevant to our understanding of bovine neosporosis are studies examining cytokine regulation in pregnancy, in particular at the materno-foetal interface (Innes *et al*. 2002). The pro-inflammatory cytokines such as IFNγ and IL-12 are involved in the generation of Th1-type immune responses that may be damaging to the pregnancy (Tangri and Raghupathy, 1993; Wegman *et al*. 1993; Entrican, 2002). The cytokine environment of the placenta favours more regulatory Th2-type cytokines such as IL-10, IL-4 and transforming growth factor beta (TGF-β) whose role is to counteract the inflammatory responses induced by the Th1-type cytokines (Entrican, 2002).

Thus the natural immuno-modulation occurring in the pregnant dam resulting in a bias towards Th2-type immune responses may compromise her ability to control *N. caninum* multiplication and the Th1-type immune responses, known to protect against *N. caninum*, may themselves be detrimental to the pregnancy (Innes *et al*. 2002, 2005*a*). A similar example of pregnancy-related changes to the immune system affecting the host-parasite relationship is seen with *Leishmania major* infection in mice where the protective immune response is also associated with a Th1-type immune response. During pregnancy there was a reduction in the IFNγ response and an increase in production of the more regulatory cytokines IL-4 and IL-10 that resulted in the pregnant mice being less able to control the infection compared to non-pregnant controls (Krishnan *et al*. 1996). Levels of progesterone in pregnant cattle also increase steadily from early to mid-gestation (Pope, Gupta and Munro, 1969) and progesterone is

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known to bias a T cell response towards a Th2 phenotype (Kalinski *et al*. 1997). These studies indicate the changing dynamics of the maternal immune response as gestation progresses that may influence the activity of the parasite within the host. Studies examining immune responses in pregnant cattle infected with *N. caninum* have noted increases in specific antibody levels around mid to late gestation that would indicate parasite activity or re-activation (Pare, Thurmond and Hietala, 1997; Stenlund *et al*. 1999; Guy *et al*. 2001; Andrianarivo *et al*. 2005). Epidemiological studies have suggested that most recorded cases of *Neospora*-associated abortion occur between 4–6 months of gestation (Anderson *et al*. 1991; Thurmond and Hietala, 1997*a*; Moen *et al*. 1998; Gonzales *et al*. 1999). The natural changes in the maternal immune response in pregnancy may therefore influence recrudescence of a persistent infection or the ability of the dam to control a new infection. Recrudescence of *T. gondii* infection is known to occur in HIV infected patients when the T cell and IFNγ response are diminished (Luft *et al*. 1984).

Studies of the local immune response at the materno-foetal interface during infection with the parasite are providing important information to help us understand the pathogenesis of disease. Recent data examining lesions in the placenta of cattle experimentally infected with *N. caninum* in early gestation has shown a strong maternal inflammatory response in those dams where foetal death had occurred (Macaldowie *et al*. 2004). Further examination of the placental tissues has shown the presence of NK cells, CD4+, CD8+ and $\gamma\delta$ T cells and IFNγ associated with foetal death, as these responses were not seen in those infected cattle carrying live foetuses or in the uninfected control cattle (Maley *et al*. 2005). It is known from other studies that direct administration of IFNγ can induce spontaneous abortion in pregnant mice (Chaout *et al*. 1990).

Therefore, while we know that Th1-type immune responses may be protective to the dam against *N. caninum* infection, this type of immune response induced in placental tissue may be highly detrimental to the foetus. These observations highlight how immune cytokines may have both a beneficial and detrimental effect on the host depending on their concentration and tissue location.

Development of foetal immunity

A further important influence determining the outcome of infection is the relative immunocompetence of the foetus at the time of challenge. The immune system of the foetus matures progressively throughout gestation (Osburn, MacLachlan and Terrell, 1982). Studies examining foetal immune responses in cattle infected with *N. caninum* in early gestation show mitogenic responses in foetal spleen and thymus cells around day 100 of gestation but there was no evidence of antigen-specific cellular or humoral immune responses at this stage (Innes *et al*. 2005*b*). Evidence of specific cell mediated and humoral immune responses occurs around 4–7 months of gestation (Andrianarivo *et al*. 2001; Almeria *et al*. 2003; Bartley *et al*. 2004). The increasing immunocompetence of the foetus as pregnancy progresses will enable the foetus to better control the parasite infection resulting in reduced disease severity.

Immunological implications of congenital infection

Further considerations are the immunological implications of congenital infection of the foetus and the disease consequence of the timing of this infection related to the immunological maturity of the foetus. In Bovine Viral Diarrhoea (BVD) disease foetuses infected before 120 days of gestation may be born infected with the virus but appear immunologically tolerant where they do not produce antibodies against the virus but are persistently infected (McClurkin *et al*. 1984). Therefore it may be possible that a similar situation also occurs with *Neospora* infection depending on when the foetus becomes infected *in utero* and whether it survives this initial encounter with the parasite. In a study reported by Innes *et al*. (2001), a group of vaccinated pregnant dams were challenged with live *N. caninum* at mid-pregnancy. Pre-colostral serum samples were collected from the calves immediately after birth and all six calves were found to be sero-negative for *N. caninum*. However, at post-mortem 6 weeks after birth, one of these six calves was found to have parasite DNA in the CNS suggestive of parasite infection in this animal. Earlier studies had also shown that naïve pregnant cattle given a low dose of *N. caninum* tachyzoites at day 70 of gestation, which did not result in abortion, gave birth to apparently healthy calves that were also sero-negative to *N. caninum* in pre-colostral blood samples but *N. caninum*-specific DNA was found in the brain and spinal cord of the calves at post-mortem (Innes *et al*., unpublished observations). In a recent paper by Kyaw *et al*. (2005) the authors observed that one of the calves in a group born to *Neospora*-infected dams had low specific antibody titres in a pre-colostral serum sample but had *N. caninum* cyst detected by immunohistochemistry and *N. caninum* specific DNA in the CNS. These observations have important implications for our understanding of disease epidemiology and may help to explain why some animals persistently transmit the parasite over successive pregnancies without developing good immunity to their congenitally acquired infection.

Therefore the dynamics of the host-parasite relationship change throughout pregnancy. Important factors influencing severity of disease in bovine

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neosporosis include the timing of the infection during pregnancy, the relative immunocompetence of the foetus and the various consequences of the maternal immune response being host protective, parasite protective and in causing immunopathology.

Vaccination strategies

As discussed in the earlier sections of this review, live vaccine preparations are more likely to stimulate appropriate cell mediated immune responses against intracellular pathogens as they more closely mimic what is happening during natural infection and the parasite antigens are presented to the immune system in the correct MHC context. There is interest in developing attenuated strains of the parasite that may be useful as vaccine preparations (Lindsay *et al*. 1999). A desirable characteristic of such vaccines may be that they do not result in the formation of tissue cysts, since in *Neospora* these may re-activate during pregnancy (Bjorkman *et al*. 1996). A highly successful commercially available vaccine to prevent toxoplasmosis in sheep utilises a live attenuated strain of *T. gondii* (Buxton and Innes, 1995). Drawbacks of live vaccines include a limited shelf-life and safety concerns therefore attention has also focused on development of killed vaccines. The major challenges in designing an effective killed vaccine against an intracellular pathogen are to select relevant antigens and to deliver these antigens to the host to stimulate appropriate and long-lasting protective immune responses.

Selection of relevant antigens: Understanding protective host immune responses may be helpful in selection of relevant antigens. Antigens recognized by immune sera and also immune T cells may prove to be useful vaccine candidates (Marks *et al*. 1998; Hemphill, 1999; Staska *et al*. 2005; Tuo *et al*. 2005). In addition, parasite antigens known to be involved in host cell invasion and parasite survival are likely to be important (Hemphill, 1999). Due to the complex interaction of the parasite and the bovine host involving different life cycle stages a killed vaccine may have to comprise a cocktail of different antigens (Innes *et al*. 2002). A recent study showed that immunisation of gerbils with a combination of the recombinant antigens, NcSRS2 and NcDG1 induced better protective immunity than when the antigens were administered singly (Cho *et al*. 2005).

Antigen delivery strategies: Live antigen delivery systems have been used to elicit immune responses against a wide range of pathogens. Recombinant virus vectors stimulate specific CMI responses against other intracellular protozoan parasites (Honda *et al*. 1998; Schneider *et al*. 1998; Oliveira-Ferreira *et al*. 2000).

Recombinant vaccinia viruses constructed to express the antigens Nc-SRS2 or NcSAG1 were able to induce protective immunity against acute *N. caninum* infection in non-pregnant mice (Nishikawa *et al*. 2001*a*) and were also able to induce protection against abortion in a pregnant mouse model (Nishikawa *et al*. 2001*b*). In both cases the best protection was achieved using the recombinant vaccinia virus expressing the NcSRS2 antigen.

Crude lysate antigen prepared from *N. caninum* tachyzoites has been tested using different adjuvant preparations in attempts to induce protective immunity in mice. The use of non-ionic surfactant vesicles as an adjuvant exacerbated encephalitis and clinical neurological disease in immunised mice (Baszler, McElwain and Mathison, 2000) and administration of antigen with Quil A or ISCOMs resulted in enhanced protection (Lunden *et al*. 2002). Administration of a crude tachyzoite lysate with ImmuMAXSR^{TM} adjuvant protected against vertical transmission of *N. caninum* in a pregnant mouse model (Liddell *et al*. 1999). Immunisation of mice using live parasites prior to mating afforded significantly better levels of protection against vertical transmission compared to mice immunised using a killed crude lysate antigen preparation (Miller *et al*. 2005). Protective immunity was also induced in mice using specific recombinant antigens, NcSRS2 incorporated into ISCOMs (Pinitkiatisakul *et al*. 2005) and NcMIC3 antigen with the Ribi adjuvant system (Cannas *et al*. 2003*a*). Immunisation with recombinant NcMIC1 resulted in a reduction in cerebral parasites (Alaeddine *et al*. 2005).

DNA vaccination: With DNA vaccines the host is injected with DNA incorporated into a plasmid containing sequences encoding the antigens of interest. An advantage of DNA vaccination is the way that the plasmid is taken up and processed by antigen presenting cells resulting in the induction of both cell mediated and humoral immune responses (Reyes-Sandoval and Ertl, 2001). Similar to strategies discussed before for vaccination against *Eimeria* parasites and *T. gondii*, cytokines and immunostimulatory DNA sequences can be co-expressed to help modulate the type of immune response required (Sakai *et al*. 2003).

Mice vaccinated intramuscularly (*im*) with a eukaryotic expression plasmid containing NcSRS2 or NcSAG1 cDNA inserts and then boosted using the recombinant antigens were better protected against *N. caninum* challenge than those mice receiving only recombinant antigen (Cannas *et al*. 2003*b*). A further study showed direct immunisation of Balb/c mice with plasmid DNA encoding NcGRA7 or NcsHSP33 protected against congenital infection with *N. caninum* (Liddell *et al*. 2003).

CpGs (oligodinucleotides) are known to activate Th1-type immune responses and pro-inflammatory

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cytokines and are thought to be useful adjuvants to enhance the immune response to vaccines against intracellular infections (Klinman, 2003; Mutwiri *et al*. 2003). Addition of the CpG adjuvant to the vaccination of mice with plasmid DNA expressing NcGRA7 significantly improved protection (Jenkins *et al*. 2004).

Killed vaccine trials in cattle

A killed *N. caninum* preparation combined with a POLYGEN™ adjuvant was used to vaccinate heifers at 35 and 63 days of gestation (Andrianarivo *et al*. 2000). The cattle were challenged with a combined *i.v*/*i.m* inoculation of live *N. caninum* tachyzoites four weeks after the second inoculation. Following vaccination, the cattle developed specific humoral and cell mediated immune responses and after challenge there was a boost to the antibody response but not to the cell mediated immune response. All of the challenged heifers, either vaccinates or controls, had infected foetuses indicating that under the challenge conditions used in this study the vaccine preparation had not successfully protected the cattle (Andrianarivo *et al*. 2000).

A commercial vaccine, Bovilis® Neoguard, Intervet comprising a killed *Neospora* tachyzoite preparation formulated with an adjuvant, SPUR® is currently commercially available in certain countries. The vaccine is administered sub-cutaneously (*sc*) on two occasions, 3–4 weeks apart in the first trimester of pregnancy. Data on the efficacy of the vaccine under field-trial conditions showed that the vaccine had around 50% protective effect against abortions occurring at 5–6 months of gestation in cattle in Costa Rica (Schetters *et al*. 2004). A similar study in dairy cattle in New Zealand showed an overall abortion rate of 4·3% in vaccinated animals compared with 5·7% in non-vaccinated animals. Analysis of the five different farms involved in the study showed considerable variation between farms with reduction in abortion due to vaccination varying from 0–54·2%. Due to the overall abortion rate being lower then expected and the uneven distribution of the samples over the five farms in the trial no definite conclusions could be drawn on the effect of vaccination on *Neospora*-related abortions (Schetters *et al*. 2004).

Concluding remarks

Recent data from controlled experimental infections of pregnant cattle is helping us to understand the complex dynamics of the host-parasite relationship in bovine neosporosis and to determine why some cattle abort their foetuses while others produce clinically healthy, albeit congenitally infected calves. Additional studies looking at induction of protective immune responses has given encouragement to the possibility of controlling the disease by vaccination. However there are still several challenges to overcome.

Mice are a convenient *in vivo* model to test out vaccination strategies and immunogenicity of candidate antigens and such studies have greatly enhanced our knowledge in this area. However, they may not be the best animal model if the target is to induce immunity in cattle to protect throughout pregnancy. The gestation period of a cow is 280 days whereas it is 20 days in a mouse. While work done in the natural bovine host is highly relevant, specific immunological reagents are more limited and it can take 12–18 months to run one vaccine trial. The most difficult aspect of setting up a challenge model in cattle to look at efficacy of candidate vaccines is to decide on an appropriate challenge. While this may be easily titrated using mouse models it would be highly costly and time consuming to do the same in cattle. Reproduction of *Neospora*-associated abortion has been achieved experimentally through intravenous, intramuscular or subcutaneous injection of tachyzoites and more recently by oral administration of oocysts (Gondim *et al*. 2004*b*). The difficulty one faces when using cattle as an *in vivo* model is selecting an appropriate challenge, using a large enough group of animals, that will cause disease in the unvaccinated controls but that will not overwhelm the immunity induced by the test vaccine.

It is also important that the vaccine is designed in such way as to induce protective immune responses without exacerbating pathology. Our knowledge of cytokine regulation during pregnancy would suggest that it may be better to vaccinate animals prior to mating as the immune response induced by the vaccine may be detrimental to the pregnancy.

In addition, further work needs to be done to determine the immunological implications of cattle becoming infected with the parasite *in utero* when their immune systems are still developing and being born persistently infected with the parasite. Does this somehow compromise their ability to develop effective immunity against *N. caninum* later in life and does this in part explain the high rates of repeated vertical transmission observed in natural infection? This would have important implications in devising a vaccination strategy as it may prove to be more efficacious to target the vaccine to naïve cattle and cull out those that are congenitally infected.

OVERALL CONCLUSIONS

Eimeria, *Toxoplasma* and *Neospora* are formidable parasites causing serious diseases in farm livestock and, in the case of *T. gondii*, people on a global scale. Commercial vaccines are available for all three pathogens but as discussed above these do not

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provide a complete or sustainable solution to the problem. There are areas of common ground in designing vaccines for these three groups of parasites such as pooling our knowledge concerning the best vaccine delivery approach to induce protective cell mediated immune responses that require appropriate processing and presentation of antigens within the correct MHC background. However, each group of parasites also has unique challenges and difficulties to overcome in order to develop novel and effective vaccines. Control of poultry coccidioses is becoming increasingly more difficult due to high levels of drug resistance and the fact that large numbers of chickens have to be used to produce the live vaccines. Challenges with developing vaccines for bovine neosporosis are to try and induce protective immunity without causing immunopathology during pregnancy and for us to understand the consequences of congenital infection on the animals' ability to mount an effective immune response to the endogenous parasite. Currently there are no toxoplasmosis vaccines for use in people. This may be due to the increased safety and legislative requirements to licence a vaccine for human use or it may be that *T. gondii* is not considered an important enough pathogen to warrant development of a human vaccine. Interestingly, new epidemiological evidence has shown that *T. gondii* was the third most common cause of food-related deaths in the USA, water-borne outbreaks have resulted in serious ocular disease and infection with the parasite has been linked with psychiatric illness (reviewed by McAllister, 2005). Therefore there may be a case to re-examine the importance of *T. gondii* as a human pathogen.

Another relevant issue is the selection of appropriate animal models and challenge dose and preparations to examine efficacy of the different candidate vaccines. Finally, there is the cost of actually getting a candidate vaccine through the necessary commercial developmental steps and onto the market which can be significant, in particular with human vaccines.

Despite these many challenges, great progress has been made working towards novel control strategies based on vaccination and we are approaching a very exciting era in understanding more about the biology of these fascinating organisms as the genome sequences of each are completed.

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