Polish Journal of Veterinary Sciences Vol. 15, No. 1 (2012), 3-9

DOI 10.2478/v10181-011-0107-7

Original article

Induction of immune responses in sheep by vaccination with liposome-entrapped DNA complexes encoding *Toxoplasma gondii* MIC3 gene

E. Hiszczyńska-Sawicka[1,3], H. Li[1], J. Boyu Xu[1], M. Akhtar[2], L. Holec-Gąsior[3], J. Kur[3], R. Bickerstaffe[1], M. Stankiewicz[1]

[1] Faculty of Agriculture and Life Sciences, Lincoln University, PO Box 84, Lincoln, Canterbury, New Zealand
[2] Immunoparasitology Laboratory, Department of Parasitology, University of Agriculture, Faisalabad, Pakistan
[3] Department of Microbiology, Chemical Faculty, Gdańsk University of Technology, Narutowicza 11/12, 80-952 Gdańsk, Poland

Abstract

Toxoplasma gondii is a parasite that has been extensively studied due to its medical and veterinary importance in terminating pregnancies. Consequently, a satisfactory vaccine is required to control its adverse effects on pregnant animals. The microneme protein, MIC3, is a major adhesion protein that binds to the surface of host cells and parasites, and is therefore a potential vaccine against *T. gondii*. The viability of MIC3 as a vaccine is investigated in this study. Sheep were injected twice, intramuscularly, with plasmids containing DNA encoding for the mature form of MIC3 protein formulated into liposomes. Control sheep were injected with an empty vector or received no injections. The injection of sheep with DNA plasmids encoding for MIC3 elicited an immune response after the first and second injections as indicated by antibody responses and the production of IFN-γ. The immune response, as measured by the IgG2 and IgG1 serum levels, was boosted after the injection of the MIC3 DNA vaccine together with high anti-MIC3 antibodies.

The results demonstrate that the intramuscular injection of sheep with a plasmid containing DNA coding for MIC3 protein induces a significant and effective immune response against *T. gondii.*

Key words: DNA vaccine, *Toxoplasma gondii*, MIC3 antigen

Introduction

Toxoplasma gondii is an intracellular protozoan parasite that invades humans and warm – blooded animals. Most of the infection (toxoplasmosis) is usually asymptomatic. Serious problems can be induced by congenital toxoplasmosis and infections in immunocompromised patients. In animals, such as sheep and goats, the congenital *T. gondii* infection can cause reproductive disorders (Buxton et al. 2007). In addition, the tissue cysts of *T. gondii* in meat of infected livestock are an important source of infection for

Correspondence to: E. Hiszczyńska-Sawicka, e-mail: ehiszczynska@yahoo.com, tel.: +64 3364 2500

humans. A live vaccine, Toxovax® (Intervet Schering-Plough, New Zealand), based on an attenuated strain of *T. gondii* is currently being used in sheep. However, such a vaccine is not suitable for humans because of the risk of potential reactivation. Thus, there is a need to develop a modern non-living vaccine with a long shelf life that is effective in farm animals and humans. Such a vaccine should provide, in a single treatment, lifelong protection. There has been significant progress in the development of vaccines over the last 15 years (Kur et al. 2009) and it may now be possible to develop a suitable vaccine against human and animal toxoplasmosis. Initial research focused on the surface antigen family (SAG) followed by research on antigens secreted by tachyzoites and bradyzoites.

Microneme proteins (MIC) play a predominant and important role in the early stages of the adhesion and invasion of *T. gondii* into host cells (Soldati et al. 2001, Tomley and Soldati 2001). Most MICs are adhesions, which show homology with adhesive domains from higher eukaryote proteins and undergo proteolytic processing of unknown biological significance during their transport to the micronemes (Cerede et al. 2002). Among the putative vaccine candidates, the micronemal protein MIC3 (90 kDa) has promising characteristics such as (i) being a potential adhesin of *T. gondii*, which is expressed in all three infective stages of *T. gondii* (tachyzoites, bradyzoites and sporozoites) and (ii) eliciting early and powerful immune responses in mice and humans (Ismael et al. 2003, Beghetto et al. 2005).

We have focused on the development of a DNA-based vaccine because such vaccines have been shown to elicit potent, long-lasting humoral and cell-mediated immunity, as well as providing protection against viral, bacterial, and parasitic infections (Beláková et al. 2007). The most common method used to deliver DNA vaccines is intramuscular injection which is known to induce a Th1-type response. The latter is generally thought to protect the host against *T. gondii* infection (Suzuki et al. 1988, Gazzinelli et al. 1996).

In the present study we have evaluated the ability of a DNA vaccine based on a plasmid encoding the mature form of the MIC3 protein, to induce an immune response in sheep.

Materials and Methods

Animals

Thirty-six two year-old Coopworth ewes, obtained from AgResearch Lincoln, New Zealand, were selected as seronegative by an ELISA (Institute Pourquier, France) latex agglutination test (Eiken, Japan) and a developed ELISA test for *T. gondii* (Pietkiewicz et al. 2004, Hiszczynska-Sawicka et al. 2003, 2005). The animals were grazed on pasture for the duration of the experiment. All animal manipulations were approved by the Lincoln University Animal Ethics Committee (AEC#73).

Experiment design

The ewes were divided randomly into three groups and treated as follows: control animals were immunized with 1mg of an empty pVAXIg plasmid per injection (group 1, n=12) or received no treatment (group 2, n=12) and the experimental animals were immunized with 1 mg of plasmid pVAXIgMIC3 (pMIC3) per injection (group 3, n= 12). All animals were injected intramuscularly in the dorsal part of the neck. Each animal received two injections (2 ml per injection) four weeks apart.

Construction of the MIC3 expression plasmid

The DNA sequence of the gene encoding a MIC3 antigen from *T. gondii* was obtained from the GeneBank database (Accession number AJ132530). Prokaryotic recombinant expressing plasmid pUET-MIC3 was used as a template for amplification of the fragment of MIC3 sequence by using a standard PCR amplification protocol (Qiagen, Australia) and primers: mic3ecov – 5' – CGA AGA TAT CCA CAT GGA CAG CCC AGA TC – 3' (forward), mic3not – 5' – GGC CGC GGC CGC CTG CTT AAT TTT CTC AC – 3' (reverse). Primers contained EcoRV and NotI sequence (underlined) to facilitate cloning. The 921bps PCR product corresponding to nucleotides from 207 to 1075 of MIC3 gene after purification was digested with both EcoRV and NotI and inserted into the EcoRV and NotI sites of pVAXIg vector (Hiszczynska-Sawicka et al. 2010) by using Fast-Link DNA ligation kits (EpiCenter) according to the manufacturer's instructions. The presence of the MIC3 gene was confirmed by restriction enzyme analysis and sequencing. The resulting plasmid pVAXIgMIC3 contains a truncated sequence of MIC3 (from 67 to 359 amino acids).

The large scale production of endotoxin-free DNA was accomplished using a commercial kit (EndoFree Plasmid Giga kit, Qiagen, Australia) according to the manufacturer's protocol. DNA for vaccination was dissolved in sterile endotoxin-free PBS (Qiagen, Australia).

In vitro expression of construct pVAXIgMIC3 in mammalian cells

Chinese Hamster ovary (CHO-K1) cells were transfected with pMIC3 or a control plasmid pVAXIg, using polycationic liposome reagent (Lipofectamine™2000, Invitrogen) as described previously (Hiszczynska-Sawicka et al, 2010). Transfected cells were grown for 24 hours before the cell pellets and supernatants were collected and analysed for transgene expression.

For immunoblots with transfected cells, SDS-PAGE and immunoblotting were performed. The separated proteins were probed with anti – *T. gondii* sheep serum. Bound antibodies were detected with peroxidase-labelled rabbit anti-sheep IgG (Pierce, USA).

CpG ODN

Unmethylated CpG ODNs 2135 – 5' – TC**GTCGTT**TGTCGTTTT**GTCGTT**–3' (Pontarollo et al. 2002) were synthesized by Invitrogen. Each sample was resuspended in PBS to a final concentration of 1 mg/ml.

Production of recombinant MIC3-His protein in *Escherichia coli*

The production of recombinant MIC3 antigen for ELISA and IFN-γ test was performed as described in Holec-Gasior et al. (2009).

The recombinant proteins were analysed by SDS-PAGE and Western-blotting. The eluted fractions were dialyzed against a phosphate-buffered saline (PBS) buffer (1% w/v NaCl, 0.075% w/v KCl, 0.14% Na2HPO4 and 0.0125% w/v KH2PO4).

DNA-liposomes-CpG complex preparation

To enhance the efficiency of plasmid DNA uptake in intramuscular injections, 1 mg of plasmid DNA was complexed with cationic lipid N-[1(2,3-di-oleoyloxy) propyl]-N,N,N-trimethylammonium chloride (DOTAP) and dioleoyl phosphatidylethanolamine (DOPE) present at a ratio of 1:1 (w/w) (ESCORT™ Transfection Reagent – SIGMA). The DNA/lipid ratio was 1:1 (w/w). 500 µg of CpG was added and the complex was then incubated for 15 minutes at room temperature.

Serology

Serum samples were collected from all the animals prior to their first injection and once per week for 4 weeks following their second (booster) injection. Serum was tested for IgG1 and IgG2 antibodies to recombinant MIC3 antigen produced in *E. coli*.

Antibody levels were measured by ELISA as described by Hiszczynska-Sawicka et al. (2010a). The ELISA plates were coated overnight at 4°C with 100 µl of recombinant MIC3 protein at 10 µg/ml in 0.1 M carbonate buffer, pH 9.6. The plates were washed with PBST (PBS pH 7.4 containing 0.05% Tween 20) and then blocked for 2 h at 37°C in 12% FBS (Invitrogen, CA, USA). Sera were diluted 1:200 in PBST buffer and 100 µl added to duplicate wells. The plates were incubated 1 h at 37°C, washed and then monoclonal mouse anti-ovine IgG1 (AgResearch, Upper Hutt, New Zealand) or monoclonal mouse anti-ovine IgG2 was added at a dilution of 1:500 and 1:100 respectively. The plates were incubated 1 h at 37°C and then washed six times. Bound antibodies were detected by using HRP conjugated rabbit anti-mouse IgG (DacoCytomation) at 1:2000 dilution for 1 h at 37°C, followed by washing and the addition of substrate. The colour development in the dark was stopped by adding 0.1 ml of 1.5 M sulphuric acid and the colour intensity was measured in a microplate reader at 492 nm. The results were expressed as an optical density (OD) ratio between OD of the sample divided by the OD of the antibody-free control.

IFN-γ test

The assay was performed with whole-blood cultures. 1 ml of heparinized blood was dispensed into multidish 48 well plates (Nunc, Denmarc) and 20 µg per ml of recombinant MIC3 antigen was then added to each well. The culture was incubated for 24 h in 5% CO_2 at 37°C in a humidified atmosphere. The supernatants were harvested and stored at -30°C until assayed for IFN-γ by using an ELISA kit (Bovigam™, Prionics AG, Switzerland) (Wood and Jones, 2001, Hiszczynska-Sawicka et al. 2010a).

Statistical analysis

The analyses were carried out using the repeated measures Anova routine in Genstat (The Guide to GenStat® Release 12, © 2009 VSN International, Edited by R.W. Payne).

Results

In vitro expression of MIC3 in CHO-K1

The ability of recombinant DNA plasmids to express the full length antigen *in vitro* was investigated in CHO-K1 cells. A DNA fragment containing the coding sequence of mature *T. gondii* MIC3 antigen was amplified and cloned into a eukaryotic expression vector (pVAXIg). Immunoblotting of CHO-K1 cells transfected with pMIC3 showed that *T. gondii* recombinant protein MIC3 was expressed *in vitro* from this plasmid vector (Fig. 1). The expression vector was designed such that the MIC3 antigen was expected to be at least partially secreted into the culture supernatant. Expression of MIC3 (~ 48 kDa) was confirmed by probing with sheep polyclonal anti-toxoplasma antiserum. A specific immunoreactive protein of approximately 48 kDa was detected in the supernatants and cells transfected with pMIC3 (Fig. 1, lines 3 and 4) which were not present in cells transfected with the control plasmid vector (Fig. 1, line 5 and 6). These same antibodies recognized the recombinant MIC3 protein produced by *E. coli* cells (Fig. 1, line 1).

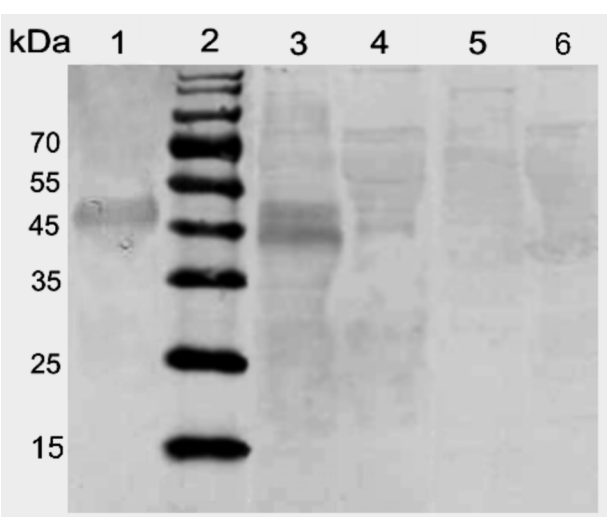

Fig. 1. Western blot analysis of recombinant MIC3 antigen expressed in CHO-K1 using sheep anti – *T. gondii* antibodies.
Lane 1: recombinant MIC3 produced in *E. coli*; Lane 2: molecular weight proteins marker (Fermentas); Lane 3: lysed cells after 24 h expression of pMIC3; Lane 4: culture supernatant after 24 h expression of pMIC3; Lane 5: control- lysed cells; Lane 6: control culture supernatant.

IFN-γ production

An in vitro stimulatory response to recombinant MIC3 antigen was demonstrated in whole blood cell cultures from each immunized sheep. The mean units of IFN-γ detected in whole blood of sheep stimulated by recombinant MIC3 antigen are shown in Fig. 2. In both control groups the changes in IFN-γ levels were not significant. In group 3, immunized with plasmid pMIC3, a significant increase in IFN gamma level was observed in weeks 2 and 5 compared to the pre-immunization level. Statistical analysis revealed differences within group 3 across the experimental time period and between the different treatment groups at particular time points (ie. weeks 2 and 5, $p<0.05$).

Antibody response to DNA immunization in sheep

In order to characterize whether a Th1 and/or Th2 response was elicited, IgG1 and IgG2 humoral responses against recombinant MIC3 protein were analyzed. In both animal control groups (immunized with the empty vector or receiving no injection) the IgG1 and IgG2 levels were the same as the pre-immunization levels.

The immunization of sheep with 1 mg of pMIC3 plasmid DNA induced a strong antibody response (Fig. 3) as illustrated by the elevated IgG1 and IgG2 levels after the first injection. The IgG1 level increased in the second week after the 1st injection, and was significantly higher than the pre-immunization levels and the levels in both control groups of animals ($p<0.01$).

The IgG2 response profile was similar to the IgG1 profile. There was a significantly elevated IgG2 level from week 2 after the 1st injection ($p<0.01$). One animal, however, produced a weak IgG1 and IgG2 response (Fig. 3).

The IgG1 and IgG2 responses were enhanced after the booster injection at week 4 and peaked at week 7. All the animals gave significant responses after the booster.

There were no significant differences between the IgG1 and IgG2 responses. However, the ratio of IgG2 to IgG1 was above 1 after the first injection and below 1 after the booster (Fig. 4), indicating that the booster injection stimulated a preferential IgG1 response.

Discussion

In the present study we have shown that DNA immunization using the MIC3 gene of *T. gondii* generates cellular and humoral responses against the MIC3 protein in sheep. We have previously shown that the intramuscular injection of 1 mg of plasmid DNA entrapped in liposomes into sheep is sufficient to trigger an immune response (Hiszczynska-Sawicka et al. 2010a,b).

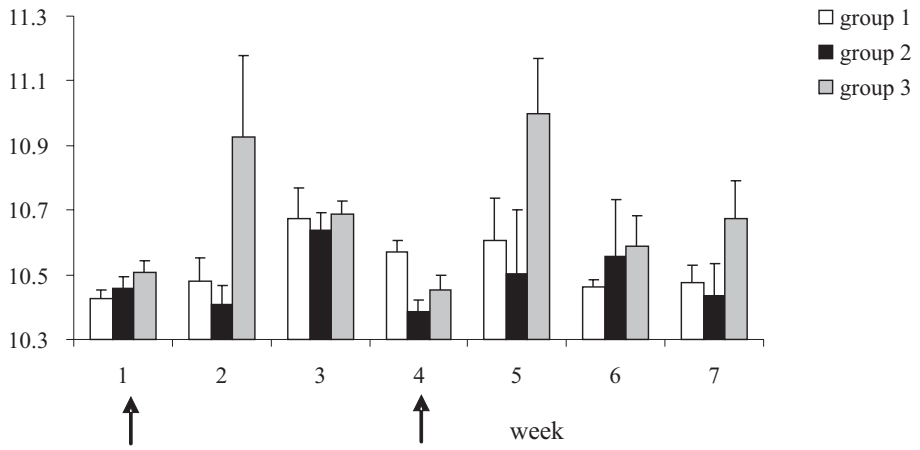

Fig. 2. Kinetics of IFN-γ production after whole blood sample restimulation with recombinant MIC3 antigen in group 1, 2 and 3 over the experimental time period. Arrows indicate time of initial immunization (week 0) and booster injection (week 4). Statistical differences between the groups are shown as * ($p<0.05$).

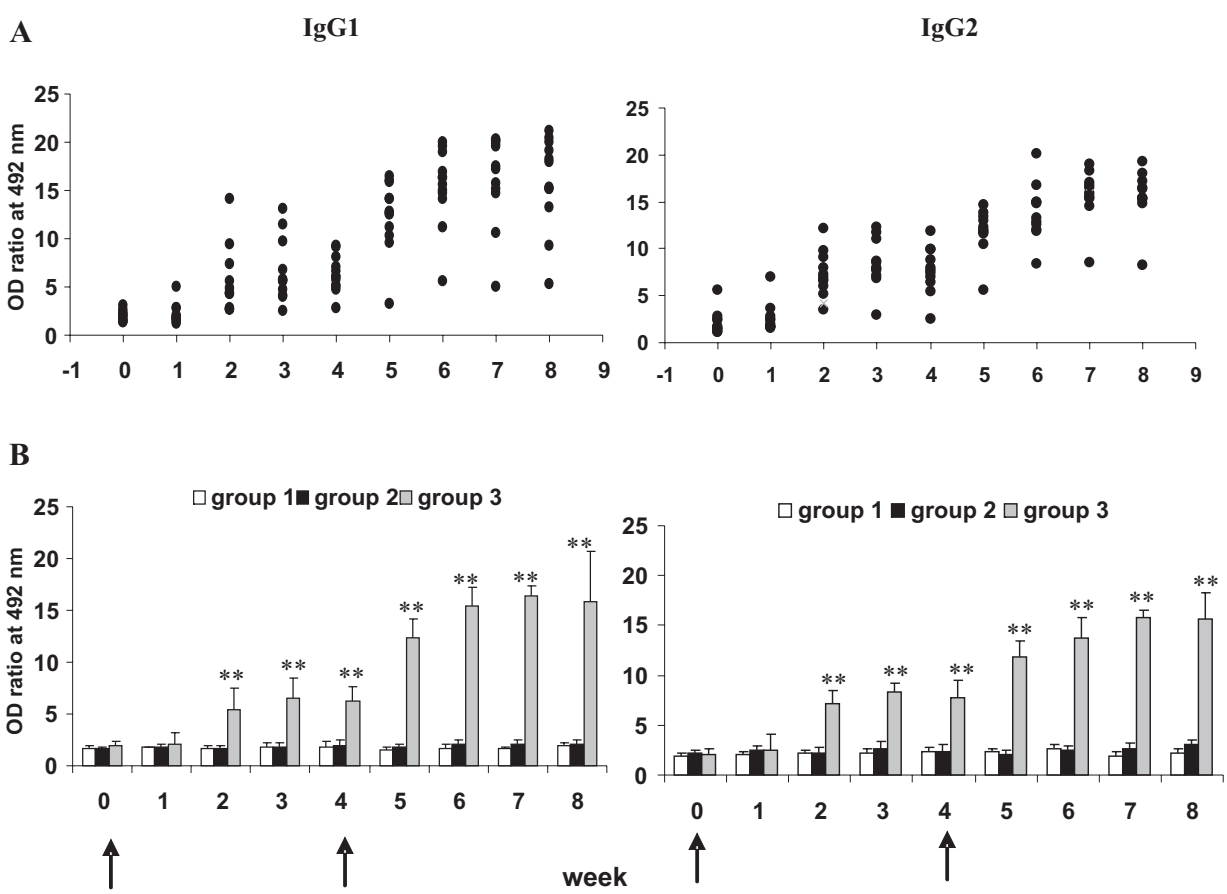

Fig. 3. Individual IgG1 and IgG2 responses (A) and specific IgG1 and IgG2 serum antibody ratio (±S.E.M.) (B) in sheep immunized with empty vector (group 1) or not immunized (group 2) and immunized with pMIC3 (group 3) following DNA immunization at weeks 0 and 4. Significant differences between group 3 and control groups are marked with stars ** ($p<0.01$).

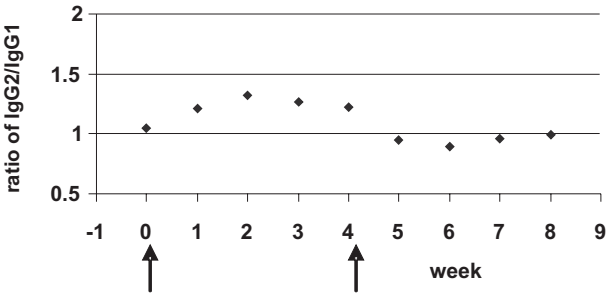

Fig. 4. Ratio of IgG2 to IgG1 in groups immunized with pMIC3. Arrows indicate time of initial immunization (week 0) and booster injection (week 4).

Ismael (2003) demonstrated in mice that a DNA vaccine encoding MIC3 antigen is a potent vaccine candidate and is capable of eliciting a strong specific immune response against *T. gondii* as well as providing significant protection against infection. Mice (CBA/J (*H-2k*)) immunized with pMIC3 developed very high anti-MIC3 IgG antibodies with a preferential production of IgG2 antibodies, suggesting the response is orientated towards a Th1-type of immune response. This was confirmed by IFN-γ production in restimulated splenocyte cultures. Xiang et al. (2009), using a DNA vaccine encoding MIC3 antigen, was also able to induce significant protection in Kunming mice. Indeed, such a vaccine significantly prolonged the survival time of mice challenged by the RH strain of *T. gondii*. In another study the oral delivery of MIC3 gene by attenuated *Salmonella typhimurium* to mice induced a Th1-type response as indicated by the significant predominance of IgG2 over IgG1 antibodies and the large production of IFN-γ (Qu et al. 2009).

In this research, we found that IFN-γ production was significantly higher in the first week after the primer or booster injections. However, the antibody response was balanced as judged by the IgG1 and IgG2 response profiles. The ratio between IgG2/IgG1 antibodies levels actually shifted towards IgG2 after the first injection and persisted at a ratio of 1 after the booster, but there were no significant differences between the IgG1 and IgG2 antibody levels.

Until recently, no vaccine that expressed MIC3 antigen had been tested in large animals such as sheep. Recently, Mevelec et al. (2010) investigated the effect of administering a mutant strain of *T. gondii* (RH strain) lacking the mic1 and mic3 genes (Mic1-3KO) to pregnant sheep. The Mic1-3KO has been previously used in mice. This resulted in the induction of a strong humoral and cellular Th1 response and a > 96% reduction in brain tissue cysts (Ismael et al. 2006). Sheep that were inoculated subcutaneously with Mic1-3KO tachyzoites showed mild febrile and IgG antibody responses. Mevelec et al. (2010) demonstrated that, after challenging pregnant sheep with the PRU strain, at least 50% of the lambs from the vaccinated sheep were viable.

Our study indicates that the intramuscular immunization of sheep with a MIC3 encoded DNA vaccine successfully triggers an immune response. All the animals developed a strong anti – MIC3 humoral response after two intramuscular injections. After the booster injection, the high levels of IgG2 and IgG1 antigen-specific antibodies and the medium level IgG2/IgG1 ratios indicate there is a mixed Th1/Th2 response. It is known that a Th-1 response is required for effective protection against a naturally occurring *T. gondii* infection and that vaccination should direct T-helper cells toward a Th1 response rather than Th-2 (Dautu et al. 2007). However, infection with *T. gondii* also stimulates humoral immunity both in the digestive tract and serum (McLeod and Mack 1986, Chardès et al. 1990). The importance of a B-cell antibody response in preventing persistent proliferation of tachyzoites in the brain and lung during the chronic phase of infection was identified by Kang et al. (2000) and Johnson and Sayles (2002). However, to verify whether the mixed immune response triggered by immunization with plasmid DNA encoding MIC3 is sufficient to protect sheep against a *T. gondii* infection needs to be investigated in future studies.

Acknowledgments

We express our gratitude to Chris Logan and the staff of the Johnstone Memorial Laboratory at Lincoln University for their help. The work described herein was funded by Ancare New Zealand.

References

Beghetto E, Nielsen HV, Del Porto P, Buffolano W, Guglietta S, Felici F, Petersen E, Gargano N (**2005**) A combination of antigenic regions of *Toxoplasma gondii* microneme proteins induces protective immunity against oral infection with parasite cysts. J Infect Dis 191: 637-645.

Beláková J, Horynová M, Krupka M, Weigl E, Raska M (**2007**) DNA vaccines: are they still just a powerful tool for the future? Arch Immunol Ther Exp (Warsz) 55: 387-398.

Buxton D, Maley SW, Wright SE, Rodger S, Bartley P, Innes EA (**2007**) *Toxoplasma gondii* and ovine toxoplasmosis: new aspects of an old story. Vet Parasitol 149: 25-28.

Cerede O, Dubremetz JF, Bout D, Lebrun M (**2002**) The *Toxoplasma gondii* protein MIC3 requires pro-peptide cleavage and dimerization to function as adhesin. EMBO J 21: 2526-2536.

Chardès T, Bourguin I, Mévélec MN, Dubremetz JF, Bout D (**1990**) Antibody responses to *Toxoplasma gondii* in

sera, intestinal secretions and milk from orally infected mice and characterization of target antigens. Infect Immun 58: 1240-1246.

Dautu G, Munyaka B, Carmen G, Zhang G, Omata Y, Xuenan X, Igarashi M (**2007**) *Toxoplasma gondii*: DNA vaccination with genes encoding antigens MIC2, M2AP, AMA1 and BAG1 and evaluation of their immunogenic potential. Exp Parasitol 116: 273-282.

Gazzinelli RT, Amichay D, Sharton-Kersten T, Grunwald E, Farber JM, Sher A (**1996**) Role of macrophage-derived cytokines in the induction and regulation of cell-mediated immunity to *Toxoplasma gondii*. Curr Top Microbiol Immunol 219: 127-139.

Hiszczyńska-Sawicka E, Brillowska-Dąbrowska A, Dąbrowski S, Pietkiewicz H, Myjak P, Kur J (**2003**) High yield expression and single-step purification of *Toxoplasma gondii* SAG1, GRA1 and GRA7 antigens in *Escherichia coli*. Prot Expr Purif 27: 150-157.

Hiszczyńska-Sawicka E, Kur J, Pietkiewicz H, Holec L, Gąsior A, Myjak P (**2005**) Efficient production of the *Toxoplasma gondii* GRA6, p35 and SAG2 recombinant antigens and their applications in the serodiagnosis of toxoplasmosis. Acta Parasitol 50: 249-254.

Hiszczyńska-Sawicka E, Li H, Xu JB, Olędzka G, Kur J, Bickerstaffe R, Stankiewicz M (**2010a**) Comparison of immune response in sheep immunized with DNA vaccine encoding *Toxoplasma gondii* GRA7 antigen in different adjuvant formulations. Exp Parasitol 124: 365-372.

Hiszczyńska-Sawicka E, Akhtar M, Kay GW, Holec-Gasior L, Bickerstaffe R, Kur J, Stankiewicz M (**2010b**) The immune responses of sheep after DNA immunization with *Toxoplasma gondii* MAG1 antigen – with and without co-expression of ovine interleukin 6. Vet Immunol Immunopathol 136: 324-329.

Holec-Gąsior L, Kur J, Hiszczyńska-Sawicka E (**2009**) GRA2 and ROP1 recombinant antigens as potential markers for detection of *Toxoplasma gondii* - specific immunoglobulin G in humans with acute toxoplasmosis. Clin Vaccine Immunol 16: 510-514.

Ismael AB, Sekkai D, Collin C, Bout D, Mevelec MN (**2003**) The MIC3 gene of *Toxoplasma gondii* is a novel potent vaccine candidate against toxoplasmosis. Infect Immun 71: 6222-6228.

Ismael AB, Dimier-Poisson I, Lebrun M, Dubremetz JF, Bout D, Mevelec MN (**2006**) Mic1-3 knockout of *Toxoplasma gondii* is a successful vaccine against chronic and congenital toxoplasmosis in mice. J Infect Dis 194: 1176-1183.

Johnson LL, Sayles PC (**2002**) Deficient humoral responses underlie susceptibility to *Toxoplasma gondii* in CD-4 deficient mice. Infect Immun 70: 185-191.

Kang H, Remington JS, Suzuki Y (**2000**) Decreased resistance of B cell-deficient mice to infection with *Toxoplasma gondii* despite unimpaired expression of IFN-γ, TNF-α, and inducible nitric oxide synthase. J Immunol 164: 2629-2634.

Kur J, Holec-Gąsior L, Hiszczyńska-Sawicka E (**2009**) Current status of toxoplasmosis vaccine development. Expert Rev Vaccines 8: 791-808.

McLeod R, Mack DG (**1986**) Secretory IgA specific for *Toxoplasma gondii*. J Immunol 136: 2640-2643.

Mévélec MN, Ducournau C, Bassuny Ismael A, Olivier M, Sèche E, Lebrun M, Bout D, Dimier-Poisson I (**2010**) Mic1-3 Knockout *Toxoplasma gondii* is a good candidate for a vaccine against *T. gondii*-induced abortion in sheep. Vet Res 41: 49.

Payne RW (**2009**) The Guide to GenStat® Release 12, Part 2: Statistics. VSN International, Hemel Hempstead.

Pietkiewicz H, Hiszczyńska-Sawicka E, Kur J, Petersen E, Nielsen HV, Stankiewicz M, Andrzejewska I, Myjak P (**2004**) Usefulness of *Toxoplasma gondii*-recombinant antigens in serodiagnosis of human toxoplasmosis. J Clin Microbiol 42: 1779-1781.

Pontarollo RA, Rankin R, Babiuk LA, Godson DL, Griebel PJ, Hecker R, Krieg AM, van Drunen Littel-van den Hurk S (**2002**) Monocytes are required for optimum in vitro stimulation of bovine peripheral blood mononuclear cells by non-methylated CpG motifs. Vet Immunol Immunopathol 84: 43-59.

Qu D, Yu H, Wang S, Cai W, Du A (**2009**) Induction of protective immunity by multiantigenic DNA vaccine delivered in attenuated *Salmonella typhimurium* against *Toxoplasma gondii* infection in mice. Vet Parasitol 166: 220-227.

Soldati D, Dubremetz JF, Lebrun M (**2001**) Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii*. Int J Parasitol 31: 1293-1302.

Suzuki Y, Orellana MA, Schreiber RD, Remington JS (**1988**) Interferon-gamma: the major mediator of resistance against *Toxoplasma gondii*. Science 240: 516-518.

Tomley FM, Soldati DS (**2001**) Mix and match modules: structure and function of microneme proteins in apicomplexan parasites. Trends Parasitol 17: 81-88.

Wood PR, Jones SL (**2001**) BOVIGAM: an in vitro cellular diagnostic test for bovine tuberculosis. Tuberculosis (Edinb) 81: 147-155.

Xiang W, Qiong Z, Li-Peng L, Kui T, Jian-Wu G, Heng-Ping S (**2009**) The location of invasion-related protein MIC3 of *Toxoplasma gondii* and protective effect of its DNA vaccine in mice Vet Parasitol 166: 1-7.