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*Immune Responses in Mice Immunised with a
Recombinant Toxoplasma gondii Antigen*

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Uppsala 2004

Report - Master of Science Programme for International Students
Faculty of Veterinary Medicine
Swedish University of Agricultural Sciences
Report no. 41
ISSN 1403-2201

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The present thesis is a partial fulfilment of the requirements for an International Master of Science Degree (MSc) in Veterinary Medicine, at the Swedish University of Agricultural Sciences (SLU), in the field of parasitology.

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Print: SLU Service/Repro, Uppsala 2004

To my parents

Abstract

Taweeanan, W. 2004. Vaccination against toxoplasmosis. Immune responses in mice immunised with a recombinant *Toxoplasma gondii* antigen. Master of Science thesis. Report no. 41, ISSN 1403-2201.

The intracellular protozoan parasite *Toxoplasma gondii* can infect a wide range of animal species, and is one of the main causes of infectious abortion and perinatal mortality in sheep. In humans, the parasite can cause abortion and congenital infection, and fatal disease in immunosuppressed patients. In sheep, toxoplasmosis can be controlled by vaccination with a live, attenuated vaccine. However, since such a vaccine has practical disadvantages and is not acceptable for use in humans, various strategies to develop an effective subunit vaccine have been explored. The major surface antigen of *T. gondii*, named SAG1, is considered as a promising vaccine candidate. Equally as important as identification of protective antigens is the choice of adjuvant. The immunostimulating complex (iscom) is an adjuvant formulation that induces both humoral and cellular immune responses that are predominantly of type 1, and therefore is likely to be effective against intracellular parasites.

The aim of the present study was to produce iscoms containing recombinant SAG1 (rSAG1) and to investigate their immunogenicity and protective capacity against *T. gondii* using a mouse model.

SAG1 expressed in *E. coli* as a recombinant protein with a hexahistidyl (His₆) tag was coupled to preformed iscom matrix (i.e. iscom particles without any antigen) using the affinity of the His₆ tag to divalent anions. The matrix contained a chelating lipid and had been loaded with Ni²⁺ ions. Analytical density gradient centrifugation revealed that a substantial proportion of the SAG1 had bound to the matrix. To investigate the immunogenicity of the rSAG1 iscoms, mice were immunised twice and the cellular immune response examined by *in vitro* stimulation of spleen cells. Cells from three of four immunised mice proliferated significantly when exposed to rSAG1, whereas cells from only one of five mice were stimulated with *T. gondii* lysate. ELISA analysis revealed high antibody titres against rSAG1 but only low levels against *T. gondii* antigens. In two subsequent challenge experiments, three groups of mice were inoculated three times with either rSAG1 iscoms, iscom matrix, or PBS. The third immunisation resulted in substantially higher antibody titres against *T. gondii* antigen. After inoculation with the virulent RH strain all mice died without any significant differences in survival time between groups ($p = 0.179$). However, when the mice were inoculated orally with tissue cysts of the Tg-SweF1 strain, significantly lower numbers of brain cysts were found in mice immunised with rSAG1 iscoms than in mice injected with PBS ($p < 0.05$).

In conclusion, although immunisation with rSAG1 iscoms did not protect mice from the lethal challenge infection, partial protection was induced as demonstrated by the reduction of brain cyst load after inoculation with an avirulent strain.

Key words: *Toxoplasma gondii*, subunit vaccine, recombinant immunogen, SAG1, iscom, immune responses

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General background

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite with an extremely wide geographic and host range. It belongs to the family Sarcocystidae, in the phylum Apicomplexa (Long, 1990). This phylum also includes other important parasites such as *Plasmodium* (the cause of malaria), *Eimeria* (the cause of chicken coccidiosis) and *Neospora* (the cause of neosporosis in cattle). Morphologically, *T. gondii* is similar to *Neospora caninum*. Before *N. caninum* was identified as a separate species, it was misdiagnosed as *T. gondii* (Dubey *et al.*, 1988). Today, there is obvious evidence, such as ultrastructural (Lindsay *et al.*, 1993; Speer *et al.*, 1999), antigenic (Howe and Sibley, 1999) and genetic differences (Brindley *et al.*, 1993), indicating that they are distinct. *T. gondii* was first described in 1908 by Nicolle and Manceaux. The name *Toxoplasma* is derived from its crescent shape (toxos = arc, plasma = form, Greek). Cats and other felid species are definitive hosts (Miller *et al.*, 1972), with humans and a wide range of warm-blooded animals acting as intermediate hosts. Toxoplasmosis is an important disease causing abortion in sheep and other animal species. It is also a cause of abortion and congenital abnormalities in humans. Moreover, in immunosuppressed individuals it can develop into a fatal disease.

Life cycle

The life cycle of *T. gondii* has an asexual and a sexual part. The asexual cycle involves two parasite stages, tachyzoite and bradyzoite. The tachyzoite is crescent-shaped, 5 μm by 2 μm in size (Smith, 1995). It can actively enter the host cell, and thereafter becomes ovoid and surrounded by a parasitophorous vacuole. The tachyzoite multiplies rapidly by endodyogeny (two daughter cells form within the mother cell) until the host cell ruptures. The released free tachyzoites penetrate new host cells, and the replication proceeds until the host dies or develops immunity to the parasite. In the latter case, the intracellular tachyzoites differentiate into bradyzoites, whereas extracellular parasites are eliminated. The bradyzoites are morphologically similar to tachyzoites, and multiply slowly within tissue cysts. These tissue cysts are found predominantly in the central nervous system and skeletal muscles. A small tissue cyst contains few bradyzoites while a large one can contain thousands. The tissue cysts represent the quiescent stage and can remain viable for the lifetime of the host. If the cysts rupture, the bradyzoites are released and become active tachyzoites that invade new host cells and then start to proliferate again. The asexual cycle can occur in both the definitive host and the intermediate host (Black and Boothroyd, 2000; Martin and Aitken, 2000).

The sexual part, which takes place only in the definitive host, begins when a non-immune cat ingests tissue cysts containing bradyzoites. The tissue cysts are digested by proteolytic enzymes in the small intestine, and bradyzoites are released. The bradyzoites penetrate into the epithelial cells and replicate asexually. After the cells break, some parasites spread via the lymphatic system and

bloodstream to other organs. Simultaneously with the multiplication and dissemination to extra-intestinal tissues, the sexual cycle, gametogony, begins in the epithelial cells of the small intestine. Found from day 3 to 15 after ingestion of tissue cysts, the gametocytes occur throughout the entire length of small intestine, but mainly in the ileum. The male microgamete enters the female macrogamete, which becomes a zygote and then develops into spherical-shaped oocyst, filled with a sporont. The oocyst is expelled into the intestinal lumen and excreted into the cat's faeces, but is not infective until it has sporulated. Sporulation takes 1 to 5 days depending on environmental factors such as aeration and temperature. The sporulated oocyst is subspherical to ellipsoidal, 11 μm by 13 μm in size, and contains 2 ellipsoidal sporocysts, each filled with 4 crescent-shaped sporozoites, 2 μm by 6 to 8 μm . The oocysts can remain infective for at least 410 days if kept at 4 $^{\circ}\text{C}$ or in the environment if not exposed to direct sunlight. Since cats usually bury their faeces, the infective oocysts can be transmitted to other animals for a year (Yilmaz and Hopkins, 1972). Widespread natural infection is not surprising since a cat may shed millions of oocysts after ingestion of an infected mouse. Oocysts can also be transported by invertebrates such as flies, cockroaches and earthworms, and by rain and snow (Dubey and Towle, 1986).

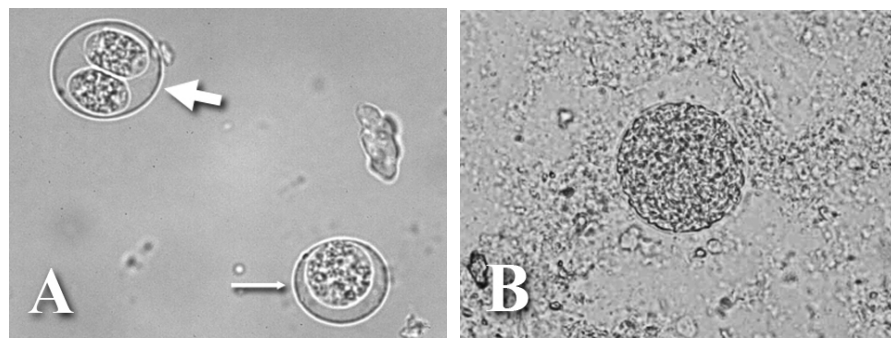


Fig. 1. *Toxoplasma gondii* oocysts (A): sporulated (large arrow) and unsporulated oocyst (small arrow). Tissue cyst in homogenised brain tissue (B). (Photo: x and S. Pinitkiatisakul)

In cats, the prepatent period (the time from infection to the shedding of oocysts) varies depending on the stage of *T. gondii* ingested. After ingesting tissue cysts, it is 3 to 10 days, 19 days or longer after ingesting tachyzoites, and 20 days or longer after ingesting oocysts (Dubey and Beattie, 1988). After a primary infection cats become immune, and do not usually shed oocysts again if reinfected. This immunity can remain for up to 6 years. However, some cats will shed oocysts again if reinfected, but then only in smaller amounts than after the primary infection (Dubey, 1995).

Vertical transmission can occur if non-immune animals become infected during the gestation period. This can lead to abortion or congenital infection. However, the susceptibility varies in different animal species. For instance, abortion due to *T. gondii* is common in sheep while it rarely occurs in cattle (Dubey and Beattie, 1988).

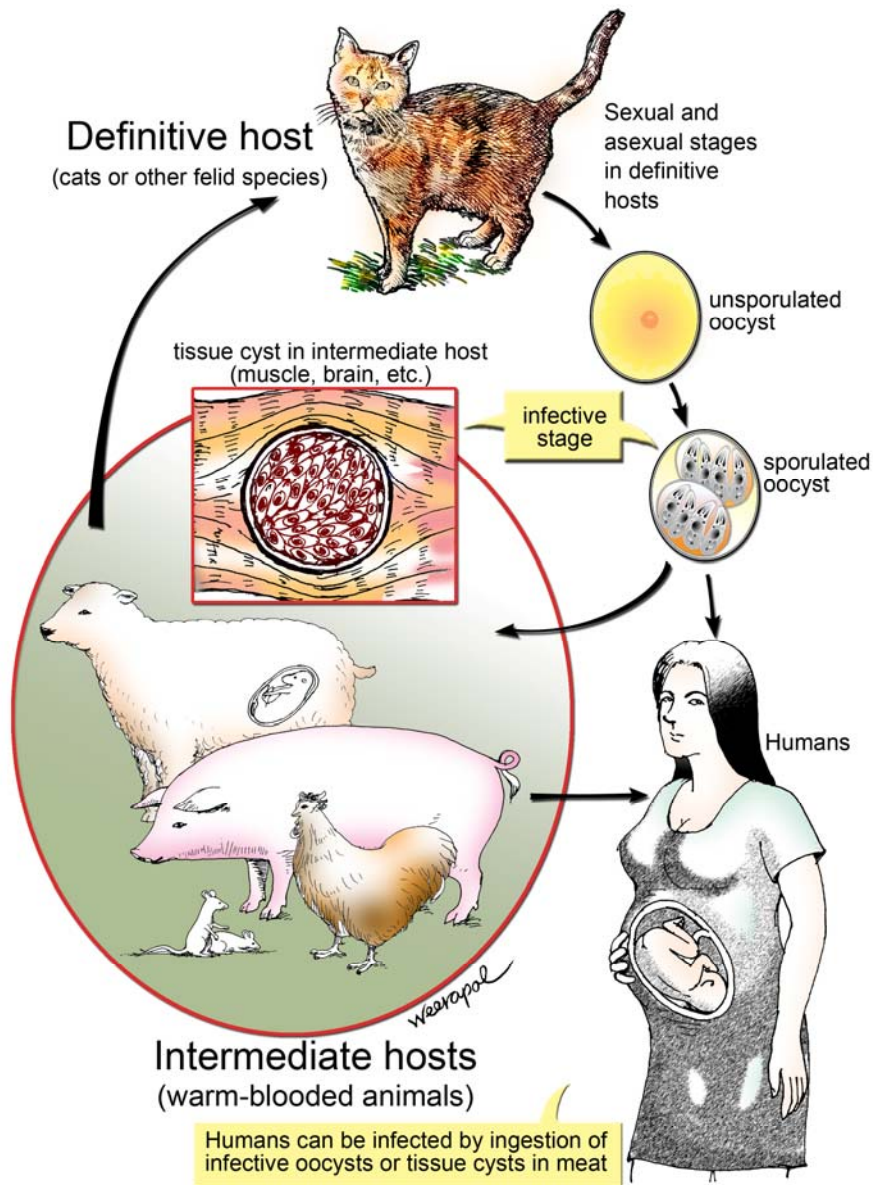


Fig. 2. Life cycle of *Toxoplasma gondii*.

Toxoplasmosis in sheep

There are several reports describing abortion in sheep caused by *T. gondii* (Dubey and Schmitz, 1981; Dubey and Welcome, 1988). Susceptible sheep can be infected by ingestion of food or water contaminated with sporulated oocysts. After lysis of the oocyst wall in the small intestine, the released sporozoites pierce the epithelial cells, become tachyzoites and start to multiply. Tachyzoites can be detected in the mesenteric lymph nodes 4 days after infection. Asexual multiplication continues, and some tachyzoites are distributed to various organs. In a pregnant ewe, the parasites can invade the gravid uterus and cause focal damage in the caruncular septa at day 10 after infection, and the degenerative changes can spread to the crypt wall and trophoblast at day 15 day (Buxton and Finlayson, 1986). The results of infection during the early period of gestation are fetal absorption or abortion, while later infection may result in stillbirth or normal but infected and immune offspring (Martin and Aitken, 2000).

In abortion cases, multifocal necrosis and calcification might be seen in the placenta. The placental cotyledons can be bright to dark red (Beverley *et al.*, 1971). Parasites might be detected in the placenta and in the fetal heart, brain, lung or liver (Dubey *et al.*, 1986b; Dubey *et al.*, 1981; Owen *et al.*, 1998). Microscopically, necroses might be found in the white matter of the fetal cerebellum and cerebrum. Focal lymphoid-cell proliferations and micronecroses might be presented in fetal kidneys, adrenals, lymph nodes or brain.

In order to diagnose toxoplasmosis in sheep, several methods can be applied. The clinical picture and history of incidence can suggest a preliminary diagnosis. Examination of aborted fetuses and maternal placentas can reveal lesions suggestive of toxoplasmosis. For confirmation, more specific methods are required, such as immunological methods, polymerase chain reaction (PCR) or isolation of the parasite by mouse inoculation (Masala *et al.*, 2003; Owen *et al.*, 1998; Wastling *et al.*, 1993). Immunohistochemical methods have been developed to detect *T. gondii* in fetuses and fetal membranes (Uggla *et al.*, 1987). Antibodies against *T. gondii* from neonatal or maternal serum can be detected by serological methods such as enzyme-linked immunosorbent assay (ELISA) (Björkman and Lundén, 1998), haemagglutination inhibition test (HI) (Chang *et al.*, 1985) or indirect fluorescent antibody test (IFAT) (Masala *et al.*, 2003; Uggla and Nilsson, 1987). These serological techniques are also useful for the epidemiological study of *T. gondii* infection.

Toxoplasmosis in other animals

While toxoplasmosis commonly affects sheep and can cause considerable economic losses, there are relatively few reports of clinical disease in other farm animals. In pigs, *T. gondii* infection has been studied because undercooked pork containing tissue cyst is considered as an important source of human toxoplasmosis. There are many reports about the prevalence of *T. gondii* infection in pigs in different countries. For example, Lundén *et al.* (2002) reported that in Sweden the seroprevalence was 3.3% in fattening pigs and 17.3% in adult swine. Gamble *et al.* (1999) investigated the prevalence of *T. gondii* infection in domestic

pigs in the New England states of the U.S.A. Using a modified direct agglutination test, they found that 47.4% of all tested sows were seropositive and 77 of 85 herds tested had at least one positive pig, which gave a herd prevalence of 90.6%. It has been demonstrated that experimental infection during pregnancy can cause transplacental transmission and abortion (Dubey and Urban, 1990; Jungersen *et al.*, 2001).

Natural outbreaks of toxoplasmosis can occur in goats. The clinical signs are mainly abortions and stillbirths. Isolation of viable parasites from placenta and aborted kids has been reported (Dubey, 1981a). Pathological lesions characteristic of *T. gondii* infection are multifocal necrosis in the placenta, and focal gliosis and calcification in the brain of aborted fetuses (Dubey *et al.*, 1986a). At the time of abortion, the antibody titre of the doe can reach up to 1:2,048 using the indirect haemagglutination (IHA) test (Dubey *et al.*, 1981). Abortion induced by experimental infection has also been investigated. In transplacentally infected kids, *T. gondii* might be isolated from the heart, brain, skeletal muscle, lungs or liver (Dubey, 1981b).

Cattle appear to be more resistant to toxoplasmosis than are sheep. There are only a few reports of abortion due to toxoplasmosis in cattle. For instance, there is a recent report of isolation of viable *T. gondii* from a naturally aborted calf (Canada *et al.*, 2002). Nevertheless, it has been shown that experimental infection can induce transplacental transmission and abortion (Stalheim *et al.*, 1980).

Toxoplasmosis in laboratory animals

Animal models for toxoplasmosis have been used for numerous reasons. For instance, they are used to study the pathology of cerebral, ocular and congenital toxoplasmosis as well as for immunological and therapeutic studies. Experimental animals can be divided into two groups according to their resistance to *T. gondii*, with rats and Old World monkeys in the resistant group and mice, hamsters, guinea pigs and New World monkeys in the sensitive group. Different animal species are used for different experimental purposes since they display a variety of pathological lesions after infection. Mice are very often used because of their small size and the availability of immunological reagents. Numerous strains of mice have been used, such as BALB/c, NMRI, Swiss-Webster, C3H, or C57BL/6j. However, although the mouse is a natural host of *T. gondii*, other species might be more suitable for study of certain aspects of toxoplasmosis (Darcy and Zenner, 1993).

Antigens of *T. gondii*

Much of the research on the antigenic structure of *T. gondii* has focused on surface membrane antigens and antigens released from secretory organelles. The tachyzoite of the RH strain is often used to study the characteristics of *T. gondii* antigens.

There are several surface antigens, and they were first named by their molecular weights, e.g. P22 for its apparent mass of 22 kDa. Currently, a naming system based on three letters related to the cellular location of the protein is used. For example, SAG1 (Surface AntiGen 1) is used for the major tachyzoite surface protein previously named P30. Likewise, other surface antigens are named SAG2 (P22) or SAG3 (P43). Other minor antigens found on the surface are SRS1 (SAG1-related sequence 1) or SRS2 (SAG1-related sequence 2) (Boothroyd *et al.*, 1998).

As a member of the Phylum Apicomplexa, *T. gondii* contains an apical complex consisting of organelles at the anterior part. These organelles include polar rings, conoid, rhoptries, micronemes and dense granules. Antigens released from the dense granules are named GRA1 (P24), GRA2 (P28), GRA4 (P41) and GRA7 (P29), and ROP1 (P66); and ROP2 (P54) are secreted from rhoptries.

The major *T. gondii* tachyzoite surface antigen, SAG1, has been widely studied. It is one of the immunodominant antigens, and it has been shown to play a role as a ligand binding to the host cell during invasion (Grimwood and Smith, 1992; Mineo and Kasper, 1994). Its potential as a diagnostic reagent has also been evaluated. SAG1 is a highly conformational antigen, and its recognition by antibodies is dependent upon the correct folding. Antiserum to native antigen does not, or does only poorly, recognise the reduced or incorrectly folded form of SAG1. For example, this has caused problems when bacterial expression systems have been used to produce recombinant SAG1 (rSAG1), while eukaryotic systems generally have worked well. The appropriate synthesis of rSAG1, expressed in CHO cells, was demonstrated as proper folding (Kim *et al.*, 1994). However, an rSAG1 fusion protein with six histidyl residues at the N-terminal was successfully expressed in *E. coli*. This recombinant protein is recognised by *T. gondii*-specific human IgG and IgM (Harning *et al.*, 1996).

Immunity to *T. gondii*

When infected with *T. gondii*, immunocompetent animals generally develop immune responses that effectively control the infection and protect against reinfection. Since *T. gondii* is an intracellular parasite, the infection is mainly controlled by cellular immune mechanisms. However, antibodies also play a role in regulation of the infection.

The cytokine gamma interferon (IFN- γ) has been identified as a major mediator of resistance against *T. gondii* (Suzuki *et al.*, 1988). IFN- γ is predominantly released by T cells and natural killer (NK) cells. It activates macrophages to destroy intracellular parasites and cytotoxic T cells to destroy *T. gondii* infected cells. It has been suggested that in case of chronic toxoplasmosis, IFN- γ protects against recurrent disease by preventing cyst rupture. This was demonstrated in mice chronically infected with the avirulent ME49 strain. When the mice were given a monoclonal antibody (mAb) against IFN- γ , encephalitis was induced and tachyzoites and *T. gondii* antigen were observed around the periphery of the tissue cysts, indicating cyst disruption (Suzuki *et al.*, 1989). Giving anti-IFN- γ mAb to mice also led to increased mortality after *T. gondii* infection (Suzuki and

Remington, 1990). IFN- γ can protect against acute toxoplasmosis without collaboration of lymphokines derived from T cells. This has been shown in athymic mice, which lack T cells. When recombinant IFN- γ (rIFN- γ) was given every other day after infection, the mice survived, but after cessation of giving rIFN- γ the mice died (Suzuki *et al.*, 1991).

The crucial role of T cells against *T. gondii* infection has been demonstrated in a number of studies. Suzuki and Remington (1988) showed that passive transfer of T cells from immune mice to naïve recipients conferred protection. Among T cell subsets, the cytotoxic CD8⁺ T lymphocytes (CTL) were found to be the most important against *T. gondii* infection (Suzuki and Remington, 1988). Adoptive transfer of CD8⁺ cells increased survival time and reduced brain cysts in challenged mice. It was also shown that these cytotoxic CD8⁺ T cells produced IFN- γ and interleukin-2 (IL-2) (Khan *et al.*, 1991; Parker *et al.*, 1991). The killing effect of CD8⁺ cells has been demonstrated *in vitro* by lysis of *T. gondii* infected mastocytoma cells (Subauste *et al.*, 1991) and macrophages (Hakim *et al.*, 1991).

Besides the cytotoxic T cells, the helper T cells are also important against toxoplasmosis. At least in mice, they are generally subdivided into T helper 1 (Th1) and T helper 2 (Th2) subpopulations according to the cytokines they produce. The Th1 cells secrete IFN- γ , IL-2 and beta tumor necrosis factor (TNF- β) whereas the Th2 cells produce IL-4, IL-5, IL-10 and IL-13 (Mosmann *et al.*, 1986; Tizard, 2000). Immunity against toxoplasmosis is associated with a Th1 type of response (Gazzinelli *et al.*, 1991).

The synergistic effects and relative importance of CD4⁺ and CD8⁺ T cells have been demonstrated by depletion of one or both subsets. Depletion of CD4⁺ T cells in naïve mice led to increased susceptibility and increased cyst burden and mortality after inoculation with an avirulent strain of *T. gondii* (Araujo, 1991). In chronically infected mice depletion of either CD4⁺ or CD8⁺ T cells did not cause any reactivation, while depletion of both subsets resulted in activation of the infection (Gazzinelli *et al.*, 1992). Protective immunity can be induced by vaccination with an avirulent non-persistent strain of *T. gondii*. In vaccinated mice, the administration of both anti-CD4⁺ and anti-CD8⁺ antibody or anti-IFN- γ completely abrogates the resistance to the infection, whereas giving anti-CD4⁺ does not affect immunity. Giving anti-CD8⁺ antibody partially reduces resistance to the infection (Gazzinelli *et al.*, 1991). Interestingly, an increase of NK cells, another important source of IFN- γ , was observed during *T. gondii* infection (Haque *et al.*, 1999).

Although cell-mediated immune responses play the essential role in immunity against toxoplasmosis, antibodies also contribute. For example, specific antibodies against SAG1 have been found to inhibit invasion of human fibroblast cells by tachyzoites (Mineo *et al.*, 1993). This was confirmed in another study showing that both monoclonal and polyclonal antibodies against rSAG1 partially inhibited the adhesion and/or invasion of host cells by *T. gondii* tachyzoites (Petersen *et al.*, 1998). In natural infection, antibodies might neutralise extracellular tachyzoites through opsonisation or complement activation.

Development of vaccines against toxoplasmosis

Numerous strategies for development of vaccines against toxoplasmosis have been investigated. Inoculation with live parasites effectively induces protection against reinfection, and one strategy is to use avirulent non-persistent strains for vaccination. One such strain is the temperature-sensitive mutant strain (ts-4), which has been shown to be non-pathogenic to mice and not cause any persistent infection (Waldeland *et al.*, 1983). It was also found that inoculated mice were well protected against lethal challenge infection. In contrast, mice inoculated with a lysate of killed *T. gondii* tachyzoites were not protected, neither when the lysate was given alone nor with an adjuvant such as Freund's incomplete or complete adjuvant (Waldeland and Frenkel, 1983). At present, there is a live commercial vaccine for sheep (Toxovax) containing the non-persistent S48 strain. The vaccine is used for prevention of ovine abortion due to toxoplasmosis (Buxton and Innes, 1995).

Generally, although live vaccines can induce protective immunity, they are not acceptable for use in humans. Subsequently, different *T. gondii* antigens have been studied using different adjuvants and antigen delivery systems. To evaluate the effect of experimental vaccines, increased survival, decreased tissue-cyst burden, prevention of congenital transmission or protection against abortion might be considered. Much work has been focused on SAG1, the major tachyzoite surface antigen, since it has been identified as one of the most promising vaccine candidates. For instance, when used with liposomes, which act as a stimulator of Th1 responses (Powers *et al.*, 1995), significant protection was induced in mice (Bülow and Boothroyd, 1991).

Presently, recombinant vaccines have been investigated, and many *T. gondii* antigens have been produced and their protective effect evaluated. For example, immunisation of mice with recombinant SAG1 (rSAG1), expressed in *E. coli* and with alum as adjuvant, resulted in significant protection against challenge with RH strain tachyzoites (Petersen *et al.*, 1998). When the protein was given together with interleukin-12 (IL-12), decreased brain cyst load and a Th1 type of immunity were recorded (Letscher-Bru *et al.*, 1998). Similarly, vaccination of mice with rSAG1, expressed in yeast *Pichia pastoris* with SBAS1c adjuvant, gave a protective effect and directed immunity towards a Th1 response (Biemans *et al.*, 1998). In guinea pigs, *P. pastoris*-expressed rSAG1 induced protective immunity and prevented congenital toxoplasmosis (Haumont *et al.*, 2000).

Also, other *T. gondii* antigens have been studied and found capable of stimulating protective immunity, for example SAG2, SRS1 (Mishima *et al.*, 2001), GRA2, and GRA5 (Zenner *et al.*, 1999).

DNA vaccines, i.e. injection of a naked DNA plasmid into the host, whose cells then express the encoded protein, have also been studied. Immunisation of mice with a plasmid encoding the SAG1 gene (p1tPASAG1) yielded almost complete protection against the virulent RH strain (Nielsen *et al.*, 1999). Reduction of brain cysts was also obtained in mice and rats immunised with a DNA plasmid encoding SAG1 (Angus *et al.*, 2000). Recently, immunisation of mice with a DNA cocktail containing plasmids encoding the SAG1 and ROP2 genes resulted in a Th1-type

response, and specific T-cell proliferation and IFN- γ production. Also, significant long-lasting protection was observed after challenge infection with the virulent RH strain (Fachado *et al.*, 2003).

Introduction to the research report

The present research report describes a vaccination experiment with rSAG1 administered together with immunostimulating complexes (iscoms). The iscom is an adjuvant formulation that was first described by Morein *et al.* (1984). The concept is to present surface membrane proteins in multimeric form by incorporating them into a matrix consisting of Quil A, phospholipid and cholesterol. Quil A is a semipurified product of the extracted crude saponin from the bark of the tree *Quillaja saponaria* Molina. The glycoside Quil A forms micelles with phospholipid and cholesterol, which appear as a matrix to which amphipathic proteins can be attached by hydrophobic interaction. The iscoms are cage-like structures 35-40 nm in diameter (Morein *et al.*, 1984).

The iscoms are highly immunogenic, and effectively induce both humoral and cellular immune responses. Some characteristics of the responses induced by iscoms are: increased major histocompatibility complex (MHC) class II expression on antigen-presenting cells; activation of T helper cells, particularly Th1; and CTL activation (Lundén *et al.*, 1996).

Iscoms containing a number of *T. gondii* antigens extracted from tachyzoites have been evaluated in a number of studies of mice and sheep. Immunisation of mice with these iscoms resulted in the induction of humoral and cellular immune responses, and partial protection against lethal infection. Characterisation of the incorporated antigens showed that two major components were the surface antigens SAG1 and SAG2 (Lundén *et al.*, 1993; Ugglå *et al.*, 1988). In another study, mice immunised with iscoms prepared from tachyzoites of two different strains of *T. gondii* developed antibodies to *T. gondii* and specific cell proliferation, but were not protected against the challenge infection (Øvernes *et al.*, 1991).

In sheep, this experimental iscom vaccine generated antigen-specific humoral and cellular immune responses (Lundén, 1995). However, in a vaccination trial no significant protection against abortion could be demonstrated (Buxton *et al.*, 1989).

Another application of the iscom concept is the preparation of antigens for use in immunoassay. For the diagnosis of *T. gondii* infection, surface antigens are generally specific while intracellular proteins can cause unspecific cross reactions. Since the iscom production procedure selects amphipathic proteins, such as surface proteins, *T. gondii* iscoms are excellent as an antigen in ELISAs (Björkman and Lundén, 1998).

As mentioned above, SAG1 has been highlighted due to its strong immunogenicity and capacity to stimulate protective immunity and iscoms appear as a suitable antigen-presenting system. Consequently, in the present research iscoms containing rSAG1 were produced and evaluated in mice. The characteristics of the iscoms were analysed as regards morphology and protein content. Antibody- and cell-mediated immunity were evaluated using *T. gondii* iscoms or a tachyzoite lysate as antigen. In addition, challenge infections with one

virulent and one avirulent *T. gondii* strain were performed to test the protective effect of the iscom vaccine.

Aims of the study

The aim of present study was to investigate the immune responses of mice after immunisation with rSAG1 coupled to iscoms, and to test the protective effect against infection with a virulent strain and a cyst-forming (avirulent) strain of *T. gondii*.

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Research report

Immune responses in mice immunised with a recombinant *Toxoplasma gondii* antigen (rSAG1) expressed in *E. coli* and coupled to iscoms

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Abstract

The present study describes immune responses in mice immunised with the major surface antigen (SAG1) of *Toxoplasma gondii*, expressed in *E. coli*. The recombinant protein (rSAG1) was coupled to immunostimulating complexes (iscoms), and the protective effect against infection with two different *T. gondii* strains was evaluated. After two immunisations, significant SAG1-specific proliferative responses were detected in three of four BALB/c mice whereas only one of five mice were stimulated *in vitro* with *T. gondii* lysate. The vaccinated mice produced high levels of antibodies against rSAG1 but only low levels against *T. gondii* antigens. Mice immunised three times and then challenged with 10⁴ tachyzoites of the virulent RH strain did not survive longer than the non-immunised control ($p = 0.179$), although high antibody titres against *T. gondii* antigens were detected. However, NMRI mice immunised three times with rSAG1 iscoms and inoculated with 30 cysts of the avirulent Tg-SweF1 strain had significantly lower brain cyst burdens than mice given PBS ($p < 0.05$). To conclude, it was found that although immunisation with rSAG1 iscoms did not protect mice from the lethal challenge infection, it induced partial protection sufficient to reduce the number of brain cysts after infection with an avirulent strain.

Key words: *Toxoplasma gondii*, subunit vaccine, recombinant immunogen, SAG1, iscom, immune response

1. Introduction

Belonging to the Phylum Apicomplexa, *Toxoplasma gondii* is an intracellular protozoan parasite with an extremely wide geographic and host range. Its life cycle includes cats and other felids as definitive hosts and humans and warm-blooded animals as intermediate hosts. Humans can become infected by ingestion of infective oocysts from cat faeces or undercooked meat containing tissue cysts. In pregnant women who encounter a primary infection, *T. gondii* can cause abortion and congenital abnormalities. Moreover, fatal infection can develop in immunocompromised individuals (Bergin *et al.*, 1992). The infection is also of veterinary importance, and can cause economic losses when non-immune pregnant sheep become infected and result in absorption of foetus, abortion or stillbirth. Sheep become infected by ingestion of infective oocysts in contaminated food or water (Dubey and Beattie, 1988). In pregnant sheep, only primary infection can cause damage, whereas reinfection does not affect the foetus (Uggla and Buxton, 1990).

To control and prevent toxoplasmosis, different strategies to develop vaccines have been applied. In the beginning, live attenuated vaccines were evaluated and found to be effective whereas killed vaccines were found to be ineffective (Waldeland and Frenkel, 1983). For sheep, a commercial vaccine is available for control of *Toxoplasma* abortion (Toxovax). It contains live tachyzoites of the 'incomplete' S48 strain of *T. gondii* (Buxton and Innes, 1995). However, although effective in sheep a live vaccine is not acceptable for use in humans since it might revert to being harmful.

During the last decade, it has been shown that experimental, killed vaccines can induce protective immunity. One of these consists of a number of tachyzoite surface proteins (Lundén *et al.*, 1993) incorporated into immunostimulating complexes (iscoms) (Morein *et al.*, 1984). It has been found to induce partial protection in mice against lethal challenge infection (Lundén *et al.*, 1993; Uggla *et al.*, 1988). In sheep, this experimental vaccine evoked specific humoral and cellular immune responses (Lundén, 1995). However, the immunity was not sufficient to give any significant protection against abortion (Buxton *et al.*, 1989). Taken together these results indicate that vaccines using iscoms as adjuvant can stimulate strong immune response, and encourage further development to improve the protective effect.

The major surface antigen SAG1 (previously named P30) of *T. gondii* tachyzoites is considered to be a main candidate antigen for vaccine development. The immunising effects of native or recombinant SAG1 (rSAG1) together with different adjuvants have been investigated by several research groups. For example, immunisation with bacterially expressed rSAG1 with alum as adjuvant was found to induce partial protection against lethal challenge infection in mice (Petersen *et al.*, 1998).

The aim of the present study was to investigate the immune responses in mice after immunisation with rSAG1 incorporated into iscoms, and to test the protective effect against infection with a virulent strain and a cyst-forming (avirulent) strain of *T. gondii*.

2. Materials and methods

2.1 Production of rSAG1

The rSAG1 was expressed in *E. coli* as a fusion protein with a hexahistidyl (His₆) tag, and purified on a Ni²⁺-chelate column (Harning *et al.*, 1996). After elution of the protein, the buffer was changed to one containing 20 mM Tris (pH 7.9), 1000 mM KCl and 20% glycerol by passage through a PD-10 column (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.2 Preparation of rSAG1 iscoms

The iscoms were prepared by the method previously described for the coupling of recombinant proteins with a His₆ tag to preformed iscom matrix, i.e. iscom particles without any antigen (Andersson *et al.*, 2001). Briefly, the iscom matrix consisting of Quil A, cholesterol, phosphatidyl choline and the chelating lipid IDA-TRIG-DSGE was loaded with Ni²⁺ by incubation in 0.1M NiCl₂. Unbound Ni²⁺ was removed by passage through a PD-10 column, and the fractions containing matrix were identified by measuring the absorbance at 214 nm (the wavelength for Quil A). After that, the matrix (corresponding to approximately 2.3 mg Quil A) was mixed with 319 µg rSAG1, incubated overnight at 4 °C and dialysed against phosphate-buffered saline (PBS). To examine whether the protein had bound to the matrix, an aliquot of the preparation was added to the top of a 10-50% sucrose gradient and centrifuged at 200,000 × g and 10 °C for 17 hours. The gradient was divided into 16 fractions, which were tested for Quil A content by spectrophotometry (214 nm) and protein content by the Bradford test (Bradford, 1976). The fractions were also analysed by enzyme-linked immunosorbent assay (ELISA) to detect rSAG1 (Andersson *et al.*, 2001). Microtitre plates were coated with serial dilutions of each fraction, and serum from mice chronically infected with *T. gondii* was used as a primary antibody. The morphology of rSAG1 iscoms was examined by electron microscopy, and the protein concentration was determined by the Bradford test. The rSAG1 iscoms were stored at -70 °C until used for the immunisation of mice.

2.3 Animals and immunisations

Female, 18-20 g, BABL/c and NMRI mice were obtained from the National Veterinary Institute, Sweden. All mice were kept in conventional cages.

The mice were immunised subcutaneously (s.c.) at the base of the tail with 0.1 ml of PBS containing rSAG1 iscoms, corresponding to a dose of 1.3 µg of protein and 10 µg of Quil A. Control mice were injected similarly with iscom matrix containing 10 µg of Quil A per dose, or with PBS. Booster immunisations were done once or twice with a 4-week interval.

2.4 Parasites

Tachyzoites of the *T. gondii* RH strain were kept in continuous *in vitro* culture in Vero cells and used for preparation of *T. gondii* iscoms (Lundén, 1995), which were used as antigens in the ELISA and Western blot. The RH tachyzoites were also used for preparation of *T. gondii* lysate, which was used in the lymphocyte stimulation test. Challenge infections were performed with RH strain tachyzoites, and with tissue cysts of the Tg-SweF1 strain (Gustafsson *et al.*, 1997) maintained in mice by passage every sixth month.

2.5 Experimental design

2.5.1 Determination of the challenge dose for the RH strain

Twelve female NMRI mice were divided into three groups, with four mice in each. The mice were inoculated s.c. with 10^4 , 10^5 or 10^6 tachyzoites of the RH strain. Clinical signs were observed twice daily, and when possible severely affected mice were killed instead of letting them die.

2.5.2 Humoral and cellular immune responses

Twenty BALB/c mice were divided into four groups, with five mice in each. Three of the groups were injected twice with either rSAG1 iscoms, iscom matrix, or PBS. The fourth group was inoculated intraperitoneally with 30 tissue cysts of the Tg-SweF1 strain, and served as an infected control group. Two weeks after the second immunisation, blood samples were collected, and the mice were killed and their spleens removed. The antibody responses were analysed by ELISA, and spleen cells were processed for analysis of cellular immune responses with the lymphocyte stimulation test (LST).

2.5.3 Challenge infection with a virulent *T. gondii* strain

Thirty-two BALB/c mice were divided into four groups, with eight mice in each. Group 1 was injected with rSAG1 iscoms and group 2 with iscom matrix. Groups 3 and 4 were injected with PBS, and served as non-immunised challenged group and non-immunised non-challenged group, respectively. Booster immunisations were given after four and eight weeks. Sera were collected three weeks after the first immunisation and two weeks after each booster. Two weeks after the third immunisation, groups 1, 2 and 3 were inoculated s.c. with 10^4 RH strain tachyzoites and group 4 with 0.1 ml cell culture medium. All mice were weighed daily from two days before the challenge infection until the end of the experiment. Clinical signs were monitored and mortality recorded twice daily. However, when possible, severely affected mice were killed to avoid unnecessary suffering.

2.5.4 Challenge infection with an avirulent *T. gondii* strain

Thirty NMRI mice were divided into three groups, with ten mice in each. Group 1 received rSAG1 iscoms, group 2 iscom matrix and group 3 PBS. Booster immunisations were given after four and eight weeks. Sera were collected three weeks after the first immunisation and two weeks after each booster. Two weeks

after the third immunisation, all mice were inoculated orally with 30 tissue cysts of the Tg-SweF1 strain. Thirty days after the challenge, the mice were killed and their brains examined, and the number of tissue cysts per brain was calculated.

2.6 ELISA

Antibody responses were analysed by ELISA as previously described (Andersson *et al.*, 2001). To examine whether the antibodies induced by the rSAG1 would react with the native protein, iscoms containing tachyzoite surface antigens including SAG1 (Lundén, 1995) were used as coating antigen. The plates were coated with *T. gondii* iscoms or rSAG1 at a concentration of 1 µg/ml. Five percent non-fat dry milk in PBS was used as blocking agent. Individual mouse sera were tested in 3-fold serial dilutions starting at 1:30. Pooled sera from mice chronically infected with the Tg-SweF1 strain of *T. gondii* was used as the positive control serum and pooled sera from normal mice as the negative control. A rabbit anti-mouse immunoglobulin (Ig) labeled with horseradish peroxidase (HRP) was used as secondary antibody at a dilution of 1:2000. All sera and the conjugate were diluted in PBS-Tween with 2% non-fat dry milk. The substrate was 1 mM tetramethylbenzidine in potassium citrate and the absorbance was read at 450 nm. Titres were calculated from interpolation of the linear part of the titration curves, and were expressed as the reciprocal of the serum dilution giving an optical density of 1.0.

2.7 Immunoblotting

SDS-PAGE and immunoblot analysis were performed to confirm the presence of rSAG1 protein in the iscom preparation, and to examine the antibody responses in vaccinated mice. *T. gondii* iscoms were used as antigen to investigate if the antibodies induced by the rSAG1 iscoms recognised SAG1 of parasite origin. The rSAG1 iscoms (0.6 µg protein per lane) and *T. gondii* iscoms (1.5 µg protein per lane) were separated under non-reducing conditions on a 12% separating gel. After the antigens were transferred to nitrocellulose membrane (45 µm pore size, Trans-Blot[®] Transfer Medium, Bio-Rad Laboratories, USA), the membranes were blocked with 5% non-fat dry milk in Tris-buffered saline (TBS). After being washed with TBS-Tween three times, the nitrocellulose membranes were incubated overnight with either pooled sera from mice immunised with rSAG1 iscom, the positive control serum, the negative control serum or the monoclonal antibody (mAb) S13 (Harning *et al.*, 1996), which were used as a marker for SAG1. The sera were diluted 1:100 in TBS-Tween with 2% non-fat dry milk. An HRP-conjugated rabbit anti-mouse Ig diluted 1:1000 in TBS-Tween with 2% non-fat dry milk was used as secondary antibody and 3,3'-diaminobenzidine tetrahydrochloride (Sigma) as substrate.

2.8 Lymphocyte stimulation test

Cell-mediated immune responses were analysed by LST. Spleen cells were suspended in RPMI 1640 supplemented with 2 mM L-glutamine, 5×10⁻⁵ mM 2-mercaptoethanol, 50 IU/ml penicillin, 50 µg/ml streptomycin and 10% fetal calf

serum. The cells were dispensed into flat-bottomed 96-well culture plates (Corning Incorporated, Corning, NY, USA) with 2.5×10^5 cells in 100 μ l per well, together with 100 μ l medium containing *T. gondii* lysate (2, 6 and 18 μ g/ml), rSAG1 (0.28, 0.83 and 2.5 μ g/ml) or concanavalin A (Con A; 10 mg/ml). Control cultures without antigen or mitogen were included, and each kind of culture was set up in triplicate. After incubation for 96 hr in 5% CO₂ at 37 °C, 0.5 mCi tritium-labeled thymidine was added to each well. After another 18 hr incubation, the radioactivity was measured as counts per minute (cpm), and the proliferative response was expressed as a stimulation index (SI) according to the formula: SI = mean cpm in stimulated cultures/mean cpm in control cultures.

2.9 Enumeration of *T. gondii* cysts in mouse brains

Each mouse brain was homogenised with a mortar and pestle in 1 ml PBS. From the homogenates, five 10 μ l samples were each placed under a 12 \times 12 mm cover slip and examined in a light microscope. The number of tissue cysts in the samples was counted and the number of tissue cysts per brain calculated.

2.10 Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the survival times after virulent challenge. ANOVA and Tukey's pairwise comparison were used for statistic comparison of the number of cysts per brain in the different groups. $p < 0.05$ was considered statistically significant.

3. Results

3.1 Characterisation of rSAG1 iscoms

The results of the analytical sucrose density gradient centrifugation are shown in Fig. 1A-C. According to the Bradford analysis and ELISA, the rSAG1 was recovered in fractions 2 and 7 from the bottom of the gradient. Also, the absorbance at 214 nm peaked in fractions 2 and 7, indicating the presence of Quil A in these fractions. Electron microscopy of the rSAG1 iscom preparation revealed the typical cage-like structures (Fig. 2).

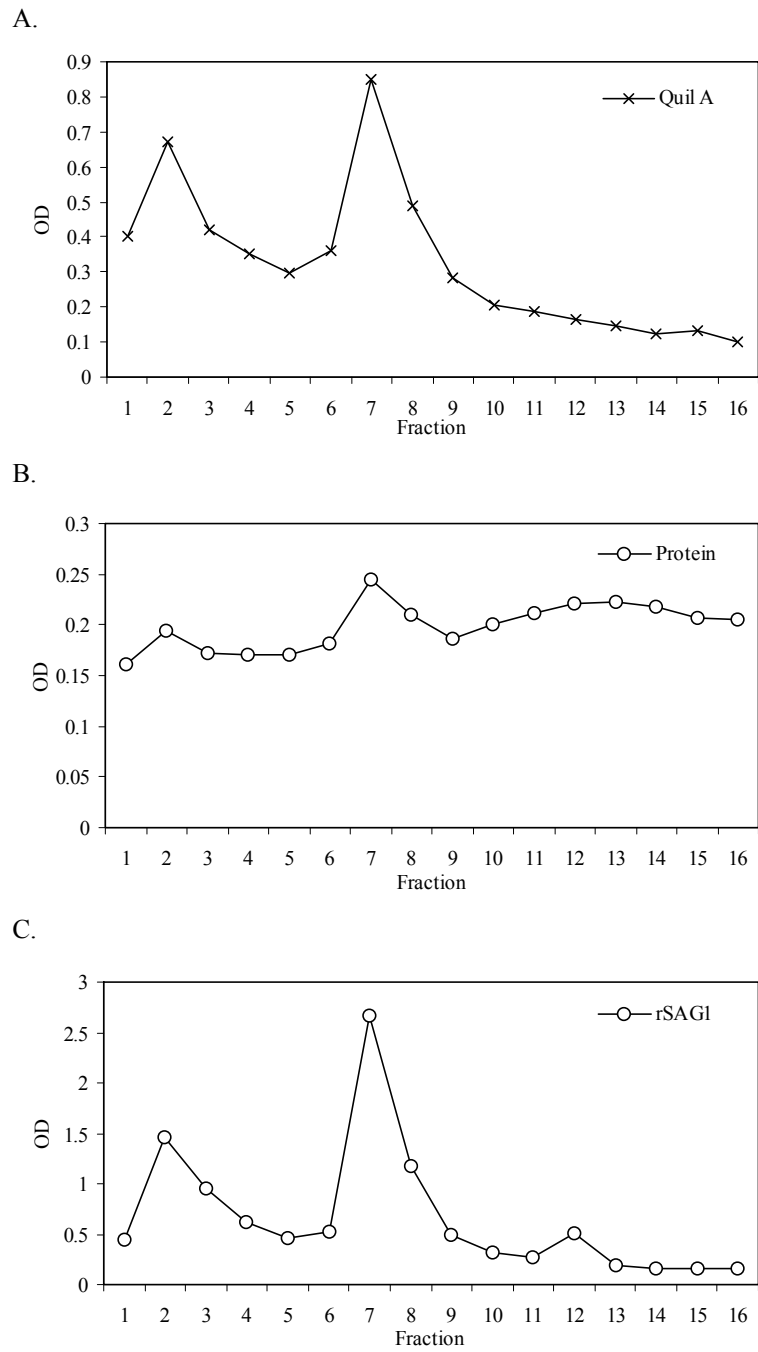


Fig.1. The rSAG1 iscom preparation was analysed by density gradient centrifugation. Each fraction was read by spectrophotometer for Quil A content (A), and analysed with Bradford test for protein content (B) and ELISA for the presence of rSAG1 (C).

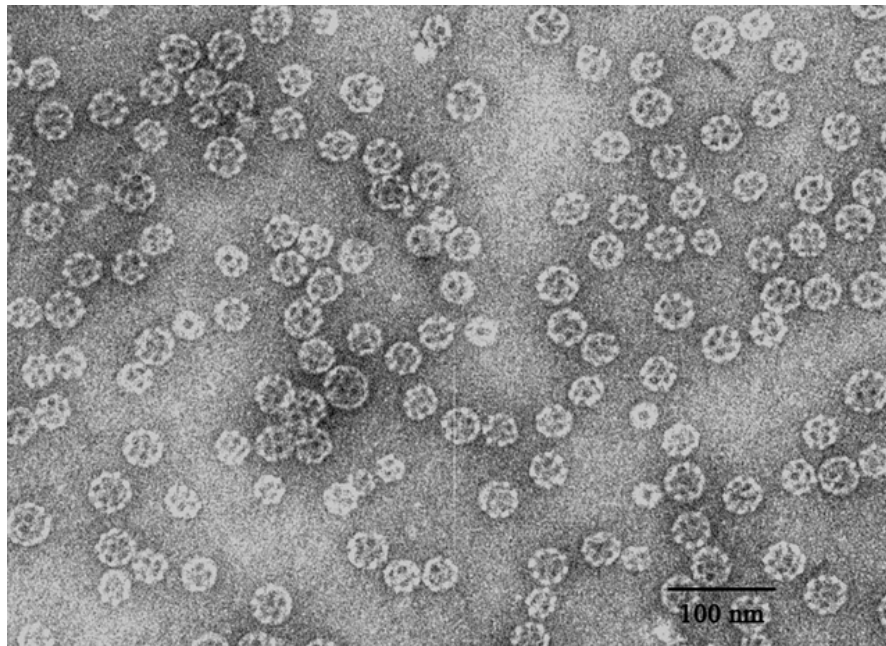


Fig. 2. Electron micrograph of rSAG1 iscoms (Photo: T. Nikkilä).

3.2 Challenge dose of the RH strain

Eight days post-infection, two of four mice given 10^4 tachyzoites died, and the other two died on day 9. The mice inoculated with 10^5 and 10^6 tachyzoites started to die on days 5 and 6, respectively, and all were dead on days 9 and 7 (Fig. 3). For the challenge experiments, the dose 10^4 was chosen since it was enough to cause lethal infection.

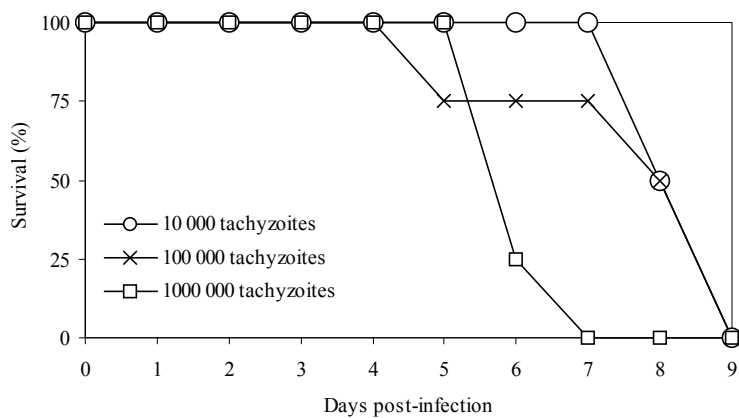


Fig.3. Percentage survival of mice inoculated s.c. with 10^4 (\circ), 10^5 (\times) or 10^6 (\square) tachyzoites of the RH strain (n = 4).

3.3 Immune responses induced by rSAG1 iscoms

The cellular immune responses were analysed by LST and an SI above 2.5 was considered to indicate significant stimulation (Kristensen *et al.*, 1982). Cells from three of four mice immunised with rSAG1 iscoms (group 1) proliferated significantly when stimulated *in vitro* with rSAG1. However, when stimulated with *T. gondii* lysate significant proliferation was induced only in cells from one of five mice. rSAG1 stimulated cells from 4 of 5 chronically infected mice (group 4) to proliferate but at a relative low level, while *T. gondii* lysate induced strong proliferative responses from this group. The level of proliferation was dose dependent and increased with the concentration of antigen (data not shown). Cells from mice given either iscom matrix or PBS (group 2 and 3) were not activated by rSAG1 or *T. gondii* lysate.

Mice immunised with rSAG1 iscoms produced high antibody levels against rSAG1 but low levels against *T. gondii* iscoms. In contrast, mice chronically infected with *T. gondii* developed high antibody titres against *T. gondii* iscoms but only low antibody titres against rSAG1. Neither group 2 nor 3 produced measurable antibody responses against any of the antigen preparation (Table 1).

Table 1. Proliferative responses and antibody titres in mice immunised with rSAG1 iscoms or infected with *T. gondii*

Group	No.	Stimulation Index (SI)			Antibody titres against	
		rSAG1 2.5 µg/ml	<i>T. gondii</i> lysate 18 µg/ml	Con A 10 µg/ml	rSAG1	Tg iscoms
1. rSAG1 iscoms	1	19.6	2.2	15.0	>65610	636
	2	20.0	1.3	308.6	>65610	267
	3	1.7	0.6	64.5	>65610	140
	4	NT	0.8	NT	>65610	119
	5	52.6	4.0	66.4	>65610	44
2. iscom matrix	1	2.2	1.2	208.0	<30	<30
	2	0.5	0.6	339.6	<30	<30
	3	0.8	0.4	113.2	<30	<30
	4	2.1	0.4	54.1	<30	<30
3. PBS	1	0.9	0.7	43.5	<30	<30
	2	0.8	0.7	46.5	<30	<30
	3	0.7	0.8	86.8	<30	<30
	4	1.2	1.5	58.6	<30	<30
	5	1.3	1.2	14.7	<30	<30
4. <i>T. gondii</i> tissue cysts	1	3.6	27.3	50.1	289	22529
	2	3.8	14.6	103.7	66	33872
	3	2.8	30.0	21.1	<30	39348
	4	5.2	69.2	74.4	54	40980
	5	1.7	14.2	26.1	226	31397
Positive serum					258	>65610
Negative serum					43	<30

3.4 Immunoblotting

Sera collected from mice immunised with rSAG1 iscoms reacted with an antigen with an approximate molecular weight of 30 kDa in both rSAG1 iscoms and *T. gondii* iscoms. The mAb S13 against SAG1, which was used to confirm the presence of rSAG1 in the rSAG1 iscoms and to identify SAG1 in the *T. gondii* iscoms, gave a similar pattern to sera from the immunised mice. The positive serum recognised a large number of antigens in the *T. gondii* iscoms, while only a single band of approximately 30 kDa in the rSAG1 iscoms was stained. The rSAG1 iscoms did not react with the negative serum, while a pale band in the *T. gondii* iscoms was stained (Fig. 4).

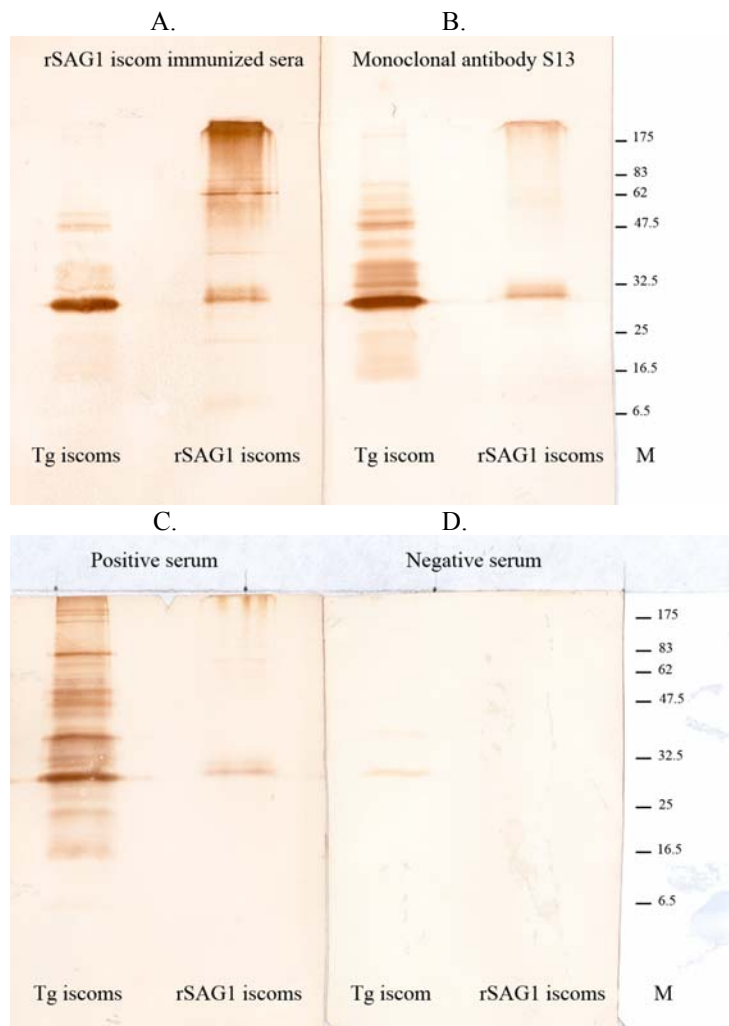


Fig. 4. Immunoblot with sera from mice immunised with rSAG1 iscoms (A), the monoclonal antibody S13 against SAG1 (B), positive serum from *T. gondii* infected mice (C) and negative serum from normal mice (D). Molecular weights (kDa) are indicated to the right.

3.5 Challenge infection with the virulent *T. gondii* strain

Since it was found in the previous experiment that two immunisations with rSAG1 iscoms only induced relatively low antibody titres against *T. gondii* antigen, the mice were immunised three times before challenge infection. However, despite increased antibody titres (Table 2), mice immunised with rSAG1 iscoms were not protected against the virulent challenge, and appeared to be as susceptible as both the non-vaccinated control and the iscom matrix group. All the infected groups started to lose weights from day 5 post-infection while the weights of the uninfected group remained at the same level as before inoculation. Moreover, all infected mice died within nine days after the challenge and, although the mean survival time for the immunised mice was slightly longer, this difference was not significant ($p = 0.179$; Table 3).

Table 2. Antibody titres of BALB/c mice immunised three times with rSAG1 iscoms before challenge infection with RH strain tachyzoites

	Antibody titres ^a against	
	rSAG1	<i>T. gondii</i> iscoms
After 1 st immunisation	216 (<30-591)	<30
After 2 nd immunisation	>65610	481(65-3447)
After 3 rd immunisation	>65610	1743(216-7107)
Positive serum ^b	226	>65610
Negative serum	<30	<30

^a Geometric mean and range, n = 8

^b From mice chronically infected with *T. gondii* Tg SweF1 strain

Table 3. Survival times of BALB/c mice challenged with RH strain tachyzoites

Group (n = 8)	Mean survival times (Days \pm SD)
1. rSAG1 iscoms	8.13 \pm 0.64
2. Iscom matrix	7.88 \pm 0.35
3. PBS	7.63 \pm 0.52

3.6 Challenge with the cyst-forming strain

The aim of this experiment was to evaluate whether immunisation with the rSAG1 iscom could decrease the cyst burden after challenge with the cyst-forming strain Tg SweF1. Antibody responses were analysed after each immunisation, and high antibody titre against both rSAG1 and *T. gondii* iscoms were demonstrated after the last immunisation (Table 4). Thirty days after infection, significantly lower numbers of brain cysts were found in mice immunised with rSAG1 iscoms than in mice given PBS ($p < 0.05$; Fig. 5). However, the number of brain cysts found in mice given iscom matrix was not significantly different from those found in the immunised group or in the PBS control group.

Table 4. Antibody titres of NMRI mice immunised three times with rSAG1 iscoms before challenge infection with Tg SweF1 strain

	Antibody titres ^a against	
	rSAG1	<i>T. gondii</i> iscoms
After 1 st immunisation	<30	<30
After 2 nd immunisation	3192 (526-80815)	(<90-546)
After 3 rd immunisation	288100 (143678-427318)	2901(373-9205)
Positive serum ^b	206	>65610
Negative serum	<30	<30

^a Geometric mean and range, n = 8

^b From mice chronically infected with *T. gondii* Tg SweF1 strain

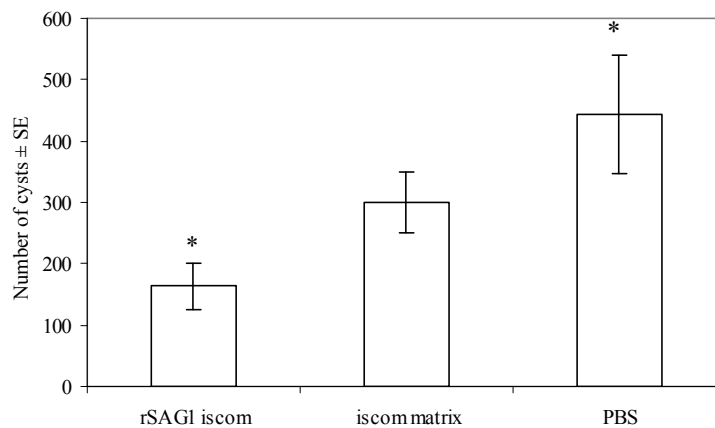


Fig. 5. Average number of tissue cyst in the brain of NMRI mice thirty days after the challenge infection. The mice were immunised with rSAG1 iscoms, injected with iscom matrix or PBS (n = 10). * = significantly different, $p < 0.05$.

4. Discussion

The immunogenicity of iscoms is dependent on the physical binding of the antigens to the iscom particle (Lövgren-Bengtsson, 1998). Analytical density gradient centrifugation is often used to examine iscom preparations. In a 10-50% sucrose gradient, iscoms and iscom matrix are typically found just below the middle of the gradient (Andersson *et al.*, 2001; Lövgren and Morein, 1988). In the present study, a large proportion of the rSAG1 appeared at this level, but a substantial amount was also detected just above the bottom of the gradient where protein aggregates are often found. According to the absorbance at 214 nm, Quil A was also present at these two levels, indicating that the protein had bound efficiently to the iscom matrix. However, the apparent presence of Quil A near the bottom of the gradient is difficult to explain. Since the spectrophotometric method used is not entirely specific for QuilA, other substances such as precipitated proteins can cause an unspecific increase in absorbance. Nevertheless, it can be

concluded that a considerable proportion of the rSAG1 had bound to the iscom matrix.

In the initial immunisation experiment it was found that the rSAG1 iscoms efficiently induced both humoral and cellular immune responses against rSAG1. However, when *T. gondii* antigen was used as antigen in the ELISA only low levels of antibodies were detected. Similarly, a *T. gondii* specific proliferative response could only be detected in one of five immunised mice. It is well known that many of the B-cell epitopes of SAG1 are conformational, and several efforts to express the protein in bacteria have failed because of incorrect folding (Burg *et al.*, 1988). The recombinant protein used in the present study has previously been shown to be properly folded (Harning *et al.*, 1996). To examine if the conformation could have been affected by coupling to the iscom matrix, the iscoms were analysed by immunoblotting. Since the monoclonal antibody S13, which is directed to a conformational epitope of the native SAG1 (Harning *et al.*, 1996), recognised the protein in the iscom preparation, incorrect folding is probably not the reason for the low responses to the native antigen.

Because of the poor immune responses against *T. gondii* antigen recorded in the initial study, an additional third immunisation dose was given before the challenge infection. This resulted in substantially increased antibody titres against the parasite antigen. However, this was not sufficient to protect the mice against challenge infection with the RH strain.

Before the challenge experiment, three doses of RH tachyzoites were tested. Subsequently, the lowest of dose, 10^4 tachyzoites, was selected since it was enough to cause a lethal infection. This can be compared to the doses used in other experiments, for example 2.5×10^4 (Lindsay *et al.*, 1990) in Swiss Webster mice, 10^5 (Petersen *et al.*, 1998) in NMRI mice and 10^5 tachyzoites in C3H and BALB/c mice (Nielsen *et al.*, 1999).

In the next challenge experiment, when a less virulent strain was used, it was found that mice immunised with rSAG1 iscoms had significantly less brain cysts than the control group given PBS only. This indicated that some degree of protective immunity against *T. gondii* infection was induced by the rSAG1 iscom.

Infection with *T. gondii* induces both antibody and cell-mediated immune responses. Since the protozoan is an intracellular parasite, the cellular immune mechanisms are the main defense of the host. The results from numerous reports illustrate that cellular immunity is more important against *T. gondii* infection than the antibody response. Passive transfer of T cells, particularly the CD8⁺ subset, from chronically infected animals can protect naïve recipients against challenge with a virulent strain (Parker *et al.*, 1991; Suzuki and Remington, 1988). CD8⁺ T cells from immune mice are cytotoxic to *T. gondii* infected cells (Hakim *et al.*, 1991), and interferon gamma (IFN- γ), produced by CD4⁺, CD8⁺ T cells or natural killer (NK) cells, is considered to be important mediator of resistance against *T. gondii* infection (Suzuki *et al.*, 1988). Neutralisation of this cytokine results in increased susceptibility to the parasite (Suzuki and Remington, 1990). The protective activity of CD8⁺ T cells is dependent on IFN- γ (Suzuki and Remington, 1990). Although the CD8⁺ T cells are crucial for protection against *T. gondii*,

CD4⁺ T cells are also important, and regulate immunity through secretion of IFN- γ and other cytokines (Gazzinelli *et al.*, 1991).

In the current study, the failure of rSAG1 iscoms to protect mice against challenge with the virulent strain could be due to poor stimulation of cellular immunity. After two immunisations, *T. gondii* specific proliferation could only be detected in one of five mice. However, the cellular response was only examined after the second immunisation, and further experiments are needed to investigate the effects of the last booster immunisation.

Several studies have been published of the immune responses and protective effect induced by immunisation with various immunogens derived from SAG1. For instance, mice given a SAG1 derived peptide developed low antibody titres, and partial protection against infection, which was demonstrated as 35% reduction in brain cyst burden (Velge-Roussel *et al.*, 1997). Reduction of the brain cyst load was also reported in mice immunised with rSAG1 plus interleukin-12 (IL-12), a mediator of Th1 responses (Letscher-Bru *et al.*, 1998). Immunisation with rSAG1, expressed in *E. coli* and with alum as adjuvant, significantly protected mice from challenge infection with RH strain tachyzoites (Petersen *et al.*, 1998). DNA vaccines based on the SAG1 gene have also been evaluated, and were in one study found to induce nearly complete protection against a lethal infection with the RH strain (Nielsen *et al.*, 1999). In another study, both reduced mortality and fewer brain cysts were observed in the immunised mice (Couper *et al.*, 2003).

The protective effect recorded as reduction of brain cyst burden in the present study leads to the suggestion that further studies should be encouraged. The immunisation dose used in the present study was based on the recommended dose of Quil A that can be used without risk of negative effects (Lövgren-Bengtsson, 1998). Each dose contained 1.3 μ g protein, which was relatively low compared to that used by, for example, Petersen *et al.* (1998) who gave four doses of 10 μ g of rSAG1. It is possible that the rSAG1 iscoms could be improved by coupling more protein to the matrix to increase the ratio of protein to Quil A, so that higher immunisation doses could be given.

Acknowledgements

This work was supported financially by the Ivar and Elsa Sandbergs Foundation, to whom we express our gratitude. The authors also thank Eva-Britt Jakubek for brilliant help with laboratory work.

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General conclusions

In general conclusion, although immunisation with rSAG1 iscoms did not protect mice from the lethal challenge infection, partial protection was induced as demonstrated by the reduction of brain cyst load after inoculation with an avirulent strain.

Acknowledgements

The studies were carried out at the Department of Biomedical Sciences and Veterinary Public Health, Division of Parasitology and Virology (SWEPAR), Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences (SLU) and National Veterinary Institute, Uppsala. Financial support was provided by. The author was granted by the Swedish Foundation for International Cooperation in Research and Higher Education (STINT). I would like to extremely express my sincere gratitude to the organizations above and to all who have contributed my work and life here.

I thankfully appreciate the following people:

Associate Professor Anna Lundén, my main supervisor, for her excellent guidance introducing me, a naïve alien, to the world of scientific research. I can completely say that she is my second parent who gives me an extremely warmth and knowledge during staying in Sweden. “A billion of words might not be enough to say thank you. You are the best and most kindly teacher in the world. Every problem would be solved by you. Actually, I cannot express my appreciation as much as I feel.”

Professor Arvid Ugglå, dean of the Faculty of Veterinary Medicine and Animal Science, previous head of SWEPAR department and my second supervisor, for the warm welcome to the research and life in department. His great and useful advice of research is still in my mind. Extremely thanks for the tasty dinner and particularly the movie ticket every Christmas. I used the former ticket, expired on the day I saw the film, for ‘The Last Samurai’.

Associate Professor Karin Östenson, course director of International Master of Science Program in Veterinary Medicine (SIPAR), SLU, for giving me a brilliant opportunity to for this course. Also thank the SIPAR staffs, **Marie Sundberg** and **Anika Rikberg** for well organized help during staying in Sweden, **Ingrid Österlundh** for being a good guide during first period in Uppsala.

Jens Mattsson, the very new head head of SWEPAR department, for all convenient facilities in the department.

Eva-Britt Jakubek, my lovely teacher, for the warm relationship and extremely helpful work. Almost for two years, I have learned not only lab but also a good mind from you. Hope you are very healthy forever. Please ask the head of department for a new CD player.

Associate Professor Prachak Puapuempoonsiri, Dean of faculty of Veterinary Medicine, Khon kaen University and **Assistant Professor Dr. Suneerat Aiumlamia** for giving me a brilliant opportunity to studying in Sweden.

The staffs of the animal house of SVA for the good taking care of my mice during the experiment. Thanks for skill of laboratory animal husbandry. You saved my life indeed.

David Morrison and **Susanne Stenlund** for checking my English, fun stories that usually make me laugh, excellent relationship and a great dinner.

Erland Ljunggren, Karin Troell, Anna Larsson, Malin Hagberg, Jackie Hrabok and **Eva Pettersson** for a word good friend. Thanks for sharing a good time together.

All of the staffs in SWEPAR department for a very nice friendship and warm welcome. I feel like SWEPAR is my second home during in Sweden.

P'Salil and **P'Tasanee Srichaiyou** for your kindness. You both are like my big brother and sister. I fell like I am not too far away from Thailand because we have a great Thai family in Uppsala supporting us several times. Please take care and I will never forget you. Best regards.

H.E.Ambassador of Thailand Poksak Nilubol and **Supajee Nilubol (P'Or)**, for taking care of Thai students, including me. Especially, a very warm and kindly relationship to join a lot of activities: Thai bazaar, CARAVAN concert, nice formal dinner, etc. Also, thank P'Or for your grand story of the novel DECEIVE which light up my mind to touch the world of writing.

P'Ubonwan (P'Aew+, a beautiful sister) and **Pear Claeson**, for all support and warm dinner several times. For help me to do something in Thailand and I appreciated.

P'On (Pornpen Birgegård) and family for being a great head of TFU. Thank for trusting me to help the TFU and for your tasty Khanom Chinese Nam Ya.

P'Uan Sunan Pinitkiatisakul for a very good friendship and excellent partner in laboratory. Thanks a lot for the Toxoplasma cyst picture despite of my responsibility, ha ha. Without you, I probably have nobody to talk and discuss of work in Thai. When I leave for Thailand, please take care yourself and our advisor the best. Don't work hard about unrelated research work!

Johnny Ericsson, the expert of computer world, for emergency help when I got trouble with my naughty skill.

All Thai students living in Uppsala for sharing an exciting experience together: Jatesada Jiwakanon, naruepon and Jaruwana Kampa – Ten ten, Suwicha Chuthatep, Arun Chanlun, Kaywalee Chatdarong, Annop and Alisara Suriyasomboon, Seri Koonjaenak, Tuempong Wongtawan, Anongnart Somchit, Pornchalit Assavacheep, Supawiwat Ponglowhapan, Sayamon Sukjumlong, Kongkiate Srisuwattanasakul, Bunlue Kornmatisuk, Sudsajjai petyim, Paisarn Tienthai, Kampon and Kosita Kaeoket. Also Thai students in Stockholm: Aumnad Phdungsilp (Joe), Wimolsiri Pridasawas (Or), Maetinee Hemrit (Bow), Win, Golf, Oei+, Nok Natinee, Mor Tee+, Terd (3rd) and Napat (Chiew).

My parents in Thailand for encouraging my inspiration to live and fight the oversea study. Hope you all are healthy and happy.

And **Wachiraporn Kampanawarawan (Jinny)** to be someone beside me even though we are so far away.