

DNA Vaccine Proposal for *Toxoplasmosis gondii*

Literature Review:

Toxoplasmosis gondii is the common, single-celled parasitic agent that causes Toxoplasmosis worldwide (Tenter, 2000). *Toxoplasmosis gondii* is zoonotic, meaning that it is naturally transmitted between humans and animals. Although *T. gondii* is likely able to infect all endothermic (warm-blooded) animals, the main concern to humans are domestic cats and animals used for meat and dairy production. It is estimated that more than 60 million people in the United States are infected with *T. gondii* (CDC, 2007) and that approximately one-third of the world's population has been exposed to the parasite (Tenter, 2000). The Center for Disease Control estimates that anywhere from 400 to 4,000 cases of congenital toxoplasmosis occur each year (CDC, 2007).

In humans, many infected individuals are asymptomatic because the human immune system is usually very effective at keeping the parasitic from causing illness. In immunocompetent people, mild symptoms like lymphadenopathy and fever sometimes occur and severe complications such as encephalitis, myocarditis, septic shock or hepatitis are rare. Infection of an immunocompetent individual usually results in lifelong immunity. Therefore, if an immunocompetent woman is infected at least 4-6 months prior to conception, the likelihood of transmission to the fetus is low because of the mother's protective immunity. However, toxoplasmosis can be a very serious problem for immunocompromised individuals and pregnant women who become infected during gestation or shortly before conception (Tenter, 2000).

For immunocompromised individuals, such as those with HIV/AIDS, Hodgkin's disease, or organ transplant and chemotherapy patients, toxoplasmosis is an opportunistic infection that poses a significant risk. Latent infection can be reactivated to cause encephalitis, which can be fatal (Singh, 2005). Cerebral abscesses (Sukhana, 2000) as well as some of the milder symptoms mentioned above may also occur. Severe encephalitis occurs in up to 40% of AIDS patient, and it is estimated that 10-30% of AIDS patients are infected with *T. gondii* (Tenter, 2000).

For pregnant women, transplacental transmission of *T. gondii* to the fetus also poses a great risk. Fetal infection occurs when tachyzoites multiply in the placenta and spread to the fetus (Jackson, 2007), and can result in abortion or a wide variety of congenital diseases. Transmission is more likely as a pregnancy progresses, but if the infection occurs in the earlier stages of pregnancy the symptoms are more severe. The incidence of prenatal infection with *T. gondii* is estimated to be anywhere from 1-120 in every 10,000 births (this does not include aborted fetus). In early stages of pregnancy about 10% of the fetal infection with *T. gondii* result in abortion or neonatal death. Another 10-23% of infected infants show signs at birth like encephalomyelitis, retinochoroiditis, convulsions, hydrocephalus, splenomegaly, hepatomegaly, fever, anemia, jaundice, or lymphadenopathy. About 12-16% of infants born die from the disease, and survivors suffer from progressive neural disorders (such as mental retardation or seizures). If transmission to an infant occurs during the third trimester, the infant is generally asymptomatic at birth. However, any child who has no symptoms at birth is still at risk to develop symptoms later in life. These symptoms usually manifest in the eye or central nervous system and include blindness, mental retardation, convulsions or other disorders. Treatment in humans is similar to treatment in cats and includes antibiotics such as Clindamycin and Trimethoprim and other potentiated sulfa drugs (Tenter, 2000).

In order to understand the transmission of *T.gondii* and how it infects the body, it is important to understand its life cycle. In herbivorous or omnivorous intermediate hosts, tachyzoites (the motile form of a zoonosis) multiply quickly in the host's cells after being ingested. The formation of tissue cysts (in which bradyzoites, the slow-growing form, multiply) is initiated by the last generation of tachyzoites. These cysts (which may persist for life in some intermediate hosts) are most often located within the eye, central nervous system, and skeletal and cardiac muscles, but may also occur in the liver, kidney, and lungs. If an individual ingests these cysts in the raw meat of an infected animal, they can then acquire the infection. If a feline definitive host ingests the cysts, asexual replication in the host is followed by the sexual phase of the *T. gondii* life cycle (gamete and oocyst formation) in the epithelium of the small intestine. Oocysts are passed into the environment with the feces and sporulate 1-5 days later, producing infectious oocysts that may be ingested. Transplacental transmission between a mother and her fetus may occur, and transmission is also possible through exposure to tachyzoites in blood, transplanted tissue, or unpasteurized milk. The incidents of transmission through meat that is undercooked is generally low because most people are aware of the risks in consuming it, but environmental transmission of oocysts is more frequent (Tenter, 2000).

As stated, members of the family Felidae are definitive hosts, therefore the cat is the only domestic animal used as a definitive host for *T. gondii*. In felines, any organ system may be infected by the parasite although most infections are mild and self-limiting. Problems with the central nervous system and eyes, as well as liver disease, fetal death and abortion may occur. The oocysts are shed for only 1-2 weeks following initial infection. The recommended treatment involves the use of antibiotics such as Clindamycin or potentiated sulfa drugs (Jackson, 2007).

It is also very important to understand the immune responses that occur with *T. gondii* infection. *T. gondii* (either as tissue cysts or oocysts) is ingested into the body. Therefore, infection occurs at a mucosal surface. After the tachyzoites are released, they invade and multiply in the intestinal epithelial cells. In the lumen of the gut, lymphocytes respond to the infection by releasing cytokines, mostly interferon- γ (IFN- γ). A cell-mediated immune response also occurs and macrophages produce interleukin-2 (IL-2) and tumor necrosis factor- α (TNF- α). Natural killers cells and T-cells are stimulated by IL-12 to secrete IFN- γ . INF- γ and TNF- α act together to mediate the killing of tachyzoites by macrophages. CD8+ T-cells are the major effector cells in the immune response to *T. gondii*. Although they work synergistically with CD4+ T-cells (helper T-cells), CD8+ cells can proliferate and secrete IFN- γ which allows for a longer period of antigen recall. CD8+ cells are also cytotoxic to infected host cells (Bhopale, 2003). The proliferation of bradyzoites is controlled by INF- γ and presentation of antigens of MHC-I (which is found on almost every nucleated cell of the body). Antibody response is also important. IgA is responsible for pathogen neutralization in mucosal surfaces, IgM is responsible for the classical pathway of complement activation, and IgG is responsible for pathogen neutralization in the tissues. Helper T-cells are also important because they cause B-cells to differentiate into either plasma cells (which produce antibodies) or memory cells. Immunocompromised individuals have a lower serologic response and lower a CD4+ count, which compromises the body's ability to fight of the infection.

Diagnosis in humans and animals may be done by detecting oocysts in the stool or performing serological testing (Jackson, 2007), like with a double-sandwich ELISA test (Hassan, 1997). IgG and IgM titers may also be helpful in diagnosing Toxoplasmosis. By looking at the

IgM/IgG ratio, one can distinguish between an acute and chronic infection. Although titers of IgM do not persist for more than a few months, titers of IgG can last for years (Malla, 2005).

The most important way to avoid the risk of toxoplasmosis is to take some key preventative measures. Milk must be pasteurized, and meat needs to be cooked thoroughly at 67°C. Freezing meat before cooking may also help reduce the risk of infection. For cat owners, it is recommended that the litter box is cleaned daily, since it takes 1-5 days for the oocysts to sporulate and become infective. Keeping your cat indoors and away from cats whose health status is uncertain helps eliminate their risk of infection. Pregnant women are advised to stay away from cat litter boxes and soil (like in a garden) that may contain cat feces. Obviously, it is also recommended that one washes their hands thoroughly after cleaning a cat litter box or preparing raw meat. It has been found that pork is most commonly infected, so extra precaution should be taken when preparing this type of meat (Jackson, 2007). Although these seem like simple solutions, they still may pose a significant dilemma for many individuals; not only would pregnant or immunocompromised veterinarians, kennel workers, pet sitters, animal control officials, etc. be limited in their ability to perform their job safely and effectively, but pregnant or immunocompromised cat owners may have to consider giving away their current cats, changing their lifestyle to be exclusively indoors, or avoiding adopting new cats (a great tragedy for cat lovers). It would be greatly beneficial for many to have access to a vaccine so that their lives, careers, and their love for cats doesn't have to come with this risk. Because of its common occurrence, Toxoplasmosis has been extensively studied (although there is still much about the biology, life cycle, and epidemiology of *T. gondii* that is unknown). There is one vaccine that is used in sheep, known as Toxovax. This vaccine is based on the live attenuated S48 strain of toxoplasmosis. It is successful at preventing toxoplasmosis in sheep, but it is expensive, causes side effects, has a short shelf life, and may revert back to its pathogenic strain (Bhopale, 2003). There have been some studies into potential vaccine options, but no vaccines have yet been developed for human use. A good market may exist for a toxoplasmosis vaccine, as it may be a great benefit to women planning on having children and immunocompromised individuals.

Vaccine Description:

Toxoplasma gondii enters the body through the mucous membranes. A vaccine that can stimulate a response in the mucous membranes would be ideal to ward off a *Toxoplasma* infection. The fact that *Toxoplasma gondii* is a parasite makes it difficult to mount an immune response against the pathogen because of its size. Its surface antigens are all structurally related but antigenically different, making it difficult to select an antigen for an immune response. Another difficulty we had to consider is that the two forms of the parasite, the tachyzoite and bradyzoite, do not have the same surface markers. However, since one of the major surface antigens of the tachyzoite stage of *Toxoplasma gondii* has shown to be important in adhesion and immune response, that surface antigen, SAG1, has been selected as the antigen to be used to stimulate a cellular immune response in vaccine recipients.

A DNA plasmid containing genes coding for the SAG1 protein has been selected as the vaccine type to be tested. Since a protein may not stimulate much of an immune response on its own, an adjuvant will be used to increase the immune response to the vaccine. Once the cells take up the plasmid they will produce the protein SAG1, which the immune system will target as the antigen. Bambuterol is a long acting beta-adrenoceptor agonist, often used in the treatment of asthma (Tenter, 2000). This adjuvant was selected because recent research has shown that inhaled vaccinations stimulate a better mucous membrane immune response. Bambuterol is a

prodrug of Terbutaline. Terbutaline relaxes the muscles of the airways and lungs and increases circulation to the areas (Tenter, 2000). The prodrug Bambuterol is a modified version that is long acting rather than fast acting, making its effect last longer. Also, being a less active form of Terbutaline, the specificity is increased. This leads to lowered chances of side effects caused by the drug acting on non specific cells. Also, a prodrug is often more easily absorbed in the body. The DNA in plasmid form will be administered intranasally by inhalation with Bambuterol. This will lead to a mucosal immune response which will result in specific antibodies to the SAG1 surface antigen, which will be produced by the cells upon taking up the plasmid.

The SAG1 antigen, as mentioned previously, is a surface protein on the protein coat of the tachyzoite stage of *Toxoplasma*. Its functions include aiding in adhesion and stimulating an immune response (Boothroyd, 2002). The immune response is directed at the tachyzoite stage of the parasite, regulating its virulence (Tenter, 2000). It is the cyst stage, or the bradyzoite stage, that is cause for concern in chronic infections. A vaccine that can protect against tachyzoite formation will halt or severely decrease the amount of bradyzoites formed, which is the ultimate goal of this vaccine. In order to stimulate an immune response against *Toxoplasma*, the antigen must be recognized and a cellular immune response must be raised. SAG1 has been selected because it is a subunit of a pathogen, meaning it will not cause adverse effects in the patient. However, the immune response expected from exposure to SAG1 would not be as apparent when the actual protein is involved in the vaccine; therefore it is the gene encoding the protein that is the vaccine. The adjuvant is used to offer extra stimulation and begin the immune response on the mucosal membrane.

SAG1 is considered to be a good choice for a vaccine because of its unique structure and the advantages the structure allows for. The structure of SAG1 is dissimilar to other known structures in its secondary structure. SAG1 is a homodimer where each subunit has two domains, D1 and D2, each of approximately the same size. The structure of these two domains has been named the SRS-fold since related SAG proteins have this structure too. The closest resemblance to the SRS-fold is found in the immunoglobulin-fold and fibronectin-III domains. However, the beta sheets in the SRS-fold are both parallel and antiparallel as opposed to the pure antiparallel of the Ig-fold. The other similar structure is found in cupredoxins, or copper binding proteins, such as azurin. Azurin has the same beta sheet arrangement but has additional beta sheets. The SRS-fold also lacks an ion binding site (Boothroyd, 2002). This unique structure makes this SAG1 protein an excellent antigen to stimulate antibodies because of its specificity. With such distinctive qualities, these antibodies would have a very low probability of recognizing self. This characteristic would also limit the occurrence of cross-reactive antibodies that recognize similar antigens. The epitope of the SAG1 protein would be on one of the two domains on either subunit. The first domain is approximately 130 amino acids in length while the second domain is approximately 120 amino acids in length. Loop structures have been identified linking the beta sheets within the domain (Boothroyd, 2002). Since epitopes are usually only a few amino acids in length, the loop structures lend more to being a possible epitopes.

Since *Toxoplasma* infection begins in the mucous membranes, the vaccination should occur here as well to switch the immunoglobulin to the IgA isotype. Once the vaccine is administered, the inhalant Bambuterol will stimulate blood flow to the airways and the mucous membranes. Bambuterol is effective in stimulating blood flow by triggering cells in the airways to release epinephrine. The plasmid will be taken up in the mucosal cells and translated into the SAG1 protein. Once the blood flow is increased in the area, there will be more macrophages present to recognize our foreign plasmid encoding the gene for SAG1. When the macrophages

have been stimulated by a DNA vaccine, they elicit an immune response against the SAG1 protein produced.

These macrophages will then secrete cytokines interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-alpha). TNF-alpha is important in inflammation, which is recruiting more macrophages and allowing other cells to seep into the infected area. The IL-12 will be secreted once the other players have arrived. Natural Killer Cells (NK cells) that are present will be amplified by IL-12 twenty to one hundred times their normal cytotoxicity. The Toll pathway is activated to increase MHC levels on dendritic cells, and complement is activated as well to help opsonize pathogen. This is the first step in an innate immune response, which is good to ward off infection, but this step is only a stepping stone to the cellular immune response for the vaccine that will give the immune system memory of the antigen. The TH1 response is the response we are looking for to build immunity to *Toxoplasma gondii*.

The dendritic cells are then sent to the lymph nodes to begin the adaptive immune response. In the lymph nodes, the dendritic cells present antigen on MHC class one and two and look for T cells with matching TCR. The T cells will be activated by recognition of MHC class one or two making them Tc, TH1, or TH2 cells. Once the NK cells and TH1 cells are activated they too begin secreting cytokines. Both of these cells secrete Interferon-gamma (IFN-gamma). IFN-gamma not only activates more macrophages, but also increases MHC class one and two levels on cells. IFN-gamma also participates in activating CD4 cells into TH1 cells. Once every cell is activated, macrophages start presenting antigen as peptides on MHC class one and two. Class one MHC will be recognized by cytotoxic T cells (Tc). However, TH1 and TH2 cells will recognize peptide on MHC class two. Depending on which MHC class is recognized, the T cells are activated. Tc cells are activated by costimulation and go out to kill infected cells. TH1 cells will be activated and released to identify infected macrophages and further activate them to kill. TH2 cells will scan B cells in the lymph node for matching BCR and activate them. An activated B cell then undergoes clonal expansion into plasma cells and memory cells. The memory cells are those that will recognize antigen if it enters the body again. Memory cells will activate plasma cells which produce antibodies. Since the antigen was first recognized on mucous membranes, the antibodies to this antigen will be of isotype IgA since it is the antibody most prevalent in mucous membranes. Antibodies will bind antigen and either aid in phagocytosis or neutralize the antigen so it cannot bind a host cell. When memory B cells have been produced they remain in the body, either in circulation or in the lymph nodes. IgA will be secreted across the mucous membranes and will be on site to bind antigen in a later infection. This will aid in a faster response since cellular immunity will be working along side innate immunity.

Discription of Immunity Assesment:

Materials and Methods-

Study participants were obtained on a volunteer basis from the Tucson Medical Center and varied in age from 18 to 57 years old. Participants were randomly divided into twelve groups, four of which were immunized with 10 μ g of foreign plasmid protein SAG1 and 0.5 μ g of the adjuvant Bambuterol (hereafter referred to as unit A), another four of which were immunized with 10 μ g of SAG1 protein alone (unit B), an additional four groups were immunized simply with 0.5 μ g of Bambuterol (unit C), and the remaining four groups were not immunized with any substance (unit D). Bambuterol was purchased from the Sigma-Aldrich Co. (2). These four groups were chosen to assess any side-effects related to the results of the immunity tests that

were not related to the vaccine and adjuvant. All substances were immunized intranasally, and participants in units A, B and C were immunized again with the same substance(s) 30 days after the initial immunization. Each study participant was analyzed for antibody and CD8+ concentrations within mucosal fluid 60 days after the initial immunization. Thereafter, each member of each group was tested every 30 days until the conclusion of the study (two years after the initial immunization date), and mucosal samples were examined using the methods described below. Oral mucosal samples were extracted from each participant one hour before the initial and secondary immunizations, and once during each 30-day analysis. Fluid was taken from participants before vaccination to assess the immunological reaction on an individual basis. Samples were stored at 4°C for 12 hours, then was centrifuged for 10 minutes and stored at -20°C until assayed for antibody concentrations using the ELISA method, or examined for total CD8+ cell concentration using flow cytometry. Both the ELISA test and the flow cytometry used SAG1 antigen. CD8+ cells were isolated from the fluid samples using flow-cytometry. Fluorochrome-attached SAG1 was then homogenized with a sample of donor-matching MHC class I tetramers for 1 hour, and was then mixed with the isolated CD8+ cells and allowed to bind for 48 hours. This sample was analyzed for bound (activated) CD8+ cells using flow cytometry.

Results-

Protection against *T. gondii* was assessed from antibody and CD8+ cell levels relative to the non-vaccinated groups (units C and D) after exposure to the bacterial plasmid, and to the vaccinated groups before and after vaccination (units A and B). Because the vaccine was given nasally, fluid samples were assayed for total mucosal IgA titers, which increased dependent of participant age over the period of the experiment in units A and B after vaccination (to 18 and 14 respectively post-immunization, and 1 and 1 respectively pre-immunization), but did not substantially increase in units C or D (2 and 1 respectively). If antibody titers remain high enough to effectively combat infection (above 5) for the period of the experiment (two years), there would indicate that the antibody response induced by this vaccine would be sufficient to prevent infection during that time period. In this case, we would expect activated CD8+ cell titers to increase in units A and B after immunization relative to pre-immunization, but to be comparatively stable in units C and D before immunization. CD8+ cell counts should also be high enough to effectively combat infection during the two-year trial, indicating that this vaccine would prevent infection.

Analysis-

Study participants were chosen from a broad age group to assess when the preferential time for vaccine administration would be. Since there was not a significant difference in vaccine efficacy relative to participant age, it can be assumed that this vaccine could be administered to all adult age groups, and immunity would last at least two years. Studies should be performed to assess exactly how long immunity lasts with this vaccine in adults. Further, since immunological memory tends to be longer lasting in children as compared to adults, additional studies need to be performed to assess how long this immunity would last in children.

Substances were immunized intranasally to stimulate mucosal cell immunity to new infections (mucosal cell immunity has a tendency to be broad and pervasive even if the infection is not). This is important because *T. gondii* typically enters via the intestinal mucosa, and patients should have broad mucosal immunity at the pathogen site of entry. As the results indicated, the adjuvant played an important role in stimulating mucosal immunity, as those

patients immunized with the vaccine and the adjuvant had higher antibody titers than those patients who were immunized with only the vaccine.

Information Sources:

Bhopale G.M. Development of a vaccine for toxoplasmosis: current status. *Microbes and Infection*. 2003. 5: 457-462.

Boothroyd, John C., Christopher Garcia, Michael E. Grigg, Xiao-lin He. 2002. Structure of the immunodominant surface antigen from the *Toxoplasma gondii* SRS superfamily. *Nature Structural Biology*. 9:606-611.

Center for Disease Control. "Toxoplasmosis." Division of Parasitic Diseases. 23 Sep 2004. 12 Feb 2007.

http://www.cdc.gov/ncidod/dpd/parasites/toxoplasmosis/factsht_toxoplasmosis.htm

Hassan M.M., Mansour S.A., Atta M., Shalaby M.M., Seksaka M.A., Awad A. The importance of detecting circulating *Toxoplasma* antigens in human cases. *Journal of the Egyptian Society of Parasitology*. 1997. 27(1): 27-34

Jackson N. "Toxoplasmosis." *Diseases of Companion Animals*. 11 Feb 2007. University of Arizona. 26 Feb 2007.

<http://microvet.arizona.edu/Courses/VSC406/index.html>

Malla N., Sengupta C., Dubey M.L., Sud A., Dutta U. Antigenaemia and antibody response to *Toxoplasma gondii* in human immunodeficiency virus-infected patients. *British Journal of Biomedical Science*. 2005. 62(1): 19-23.

Singh M.P., Dubey M.L., Sud A., Malla N. Antibody response to *Toxoplasma gondii* in saliva samples from human immunodeficiency virus-infected patients. *British Journal of Biomedical Science*. 2005. 62(2): 81-84

Sukhana Y., Chintana T., Lekkla A. *Toxoplasma gondii* antibody in HIV-infected persons. *Journal of the Medical Association of Thailand*. 2000. 83(6): 681-684.

Tenter A.M., Heckeroth A.R., Weiss L.M. *Toxoplasma gondii*: from animals to humans. *International Journal for Parasitology*. 2000. 30: 1217-1258.

2. <http://www.sigmaaldrich.com/catalog/search/ProductDetail/SIGMA/B8684>

3. http://www.biodesign.com/results_2.asp?searchbox=RH%20strain&page=1&group=9