

Intranasal Ag2/PRA Vaccine to Coccidioides immitis for Pet Canines

Background Information:

Coccidioidomycosis, the condition known as San Joaquin Valley Fever, is caused by two species of fungi known as *Coccidioides immitis* and *C. posadasii*. These fungi are dimorphic, existing in the mycelial phase in the soil, and existing in the spherical/endospore phase upon infection of a host (Hector et al., 2005). The disease is classified as endemic in the Lower Sonoran Life Zone, which consists of the Southwest region of the United States, Mexico, Central and South America (Cox et al., 2004). The disease caused by *C. immitis* and *posadasii* ranges from a minor pulmonary infection causing limited or no symptoms to a serious disseminated disease that can infect internal organs, bones, and the central nervous system.

C. immitis and *posadasii* live in the alkaline sandy soil in climates with little rainfall and high temperatures. Upon drying of the soil, the fungus buds off to form arthrospores that become aerosolized via soil disruptions such as wind or human/animal activities. The inhalation of the arthroconidia leads to the transformation of the fungus to its endosporulating parasitic form within host tissue (Cox et al., 1988). Symptoms of infection can include fever, coughing, weight loss, decreased energy, decreased appetite, enlarged lymph nodes, abscesses, lameness, neck and back pain, and seizures. These symptoms generally occur within 3 weeks of infection. Infected hosts are not contagious and the disease is not considered zoonotic.

Valley Fever is a condition that can affect a variety of mammals, including: humans, canines, felines, non-human primates, cattle, horses, and many others. The research being performed in this experiment will focus on development of a vaccine for canines since they are highly susceptible to *Coccidioides* infections due to their frequent exposure to arthrospores through sniffing and digging. Diagnosis of canines is generally done by performing an antibody titer, which is designed to measure both IgG and IgM specific for *Coccidioides*. IgM presence indicates an early infection, while the presence of IgG is indicative of a longer infection and a higher probability for dissemination (Cox et al., 2004). In general terms, high titer antibody response to *Coccidioides* infections indicates a higher probability for a more serious infection (Fierer et al., 2006). Upon diagnosis of Valley Fever in canines, veterinarians treat the disease using anti-fungal drugs such as: fluconazole, ketaconazole, or intraconazole. These drugs are administered until antibody production is halted. This can take months or even years to accomplish.

The severity of a *Coccidioides* infection is highly dependant on the immune response of the host. Less severe or asymptomatic infection is associated with high levels of T cell cytokines, such as interferon γ (IFN- γ) and interleukin 2 (IL-2) (Ampel et al., 2000). Dendritic cells are the primary antigen-presenting cells responsible for activating both T and B cells during an infection. Upon infection, dendritic cells are sent to inflammatory sites to bind pathogen and return to lymph nodes via the lymphatic system, where T and B cells are stored. Pathogen binds dendritic cells by using Toll-like receptors which leads to the production of various chemokines resulting in the maturation

of dendritic cells. Mature dendritic cells induce adaptive immunity necessary for fighting future *Coccidioides* infections.

The major cell of the immune system associated with the production of the necessary cytokines for immunity to Valley Fever is CD4+ T helper 1 cells. It has been shown in mice that CD4+ T cells were sufficient in providing immunity to *C. Immitus* infections. The same study also showed that mice without CD4+ cells could also be protected from infection through their CD8+ cytotoxic T cells (Silva et al., 2006). Although the primary function of CD8+ T cells is to lyse cells showing pathogenic peptide on their MHC class 1, they can also produce cytokines such as Tumor Necrosis Factor α (TNF- α) and IL-2.

The possibility of a creating a vaccine for *Coccidioides* is promising, primarily because hosts recovering from a less severe or asymptomatic infection exhibit immunity to subsequent infections (Hector et al., 2005). Countless efforts to create a vaccine have been attempted, although currently there is no vaccine licensed for use in canines for *C. immitus* or *posadasii*. Previous vaccine trials involved mechanisms using a whole killed spherule. These approaches were ineffective because a tolerable dose of the vaccine does not produce sufficient immunity (Silva et al., 2003). Numerous vaccine efforts have been attempted using viable cells, non viable cells, and various cell-derived antigens (Cox et al., 2004).

Recently, vaccines have been designed using adjuvants specific for *Coccidioides* which bind dendritic cells, thus stimulating production of TH1 cells. The problem with using peptides, protein subunits, and DNA plasmids is that they do not confer an immune response on single handedly. Therefore, it is important to ensure that the peptide that binds APCs most effectively is selected. Another vaccine showing promise utilizes attenuated *cholera* strain 638 to produce the *C. immitus* Ag-2/PRA present on the cell wall of fungal spherules. Patients infected with *C. immitus* have been shown to produce both antibodies and Th1 cells to protect against Ag-2/PRA, making it a viable candidate for a vaccine (Silva et al., 2003). Recent vaccine efforts have also noted the importance of delivering the vaccine via the mucosal route (intranasal vaccine) (Cox et al., 2004). This is vital because it delivers the vaccine directly to cells in the upper and lower respiratory tract. This is significant because mechanism of infection of *C. immitus* and *pasadasii* results from the inhalation of spores through the respiratory tract.

The vaccine being tested in this experiment uses the Ag2/PRA antigen to induce both cellular and humoral immunity to *C. immitus*. In order to enhance the effect of the Ag-2/PRA and to create a more effective vaccine, the adjuvant CpG DNA is used. CpG DNA is responsible for activating B-cells, T-cells, NK cells, monocytes, macrophages, and dendritic cells, increasing their production of cytokines such as IL-12, IFN- γ . In addition, CpG DNA increases expression of CD86 (B7-2) on dendritic cells, thus enhancing T-cell activation when compared to unstimulated dendritic cells (Hartmann *et al*, 1999). Increased expression of CD86 allows for the co-stimulatory response needed to activate T-cells and induce cell mediated immunity.

Coccidioides immitis Vaccine:

In order to make a vaccine that sufficiently protects the body against *Coccidioides immitis*, a strong T-helper 1 cell response needs to be stimulated, along with a B cell

response. The best way to stimulate such a response from the body comes from using an intranasal DNA vaccine, more specifically, the Ag-2/PRA antigen (Benitez *et al*, 2003). A simple live vaccine consisting of viable *Cocci immitis* spores would not surmount an effective immune response. Although injection of such a vaccine into an animal would effectively lower the amount of fungus in the body; the side effects are great, leading to viable organisms replicating at site of vaccination. Additionally, using a mutant strain of a viable cell leads to reacquisition of virulence. The use of a vaccine containing non-viable cells of *C. immitis* ends up causing excessive toxicity in the body.

When injecting the body with Ag-2/PRA, there is a drastic drop in fungal spores from infection, post vaccination, with few to no side-effects. Research started on this vaccine with C-ASWS, a high-molecular-weight polysaccharide protein from the wall of the spores, which induces a good response to *C. immitis*. Identical to this protein is another protein commonly called Antigen-2 protein, which is rich in proline and elicits a strong response when inserted into a plasmid, pVR1012. The only disadvantage to such a vaccine is whether it will produce an immune response in animals with a genetic predisposition to infection with *C. immitis* (Cox and Magee, 2004).

However, Ag-2/PRA alone will not elicit the strong enough response alone; an adjuvant is needed. For a vaccine against *Coccidioides immitis*, CpG DNA works out to be the best adjuvant (Fierer *et al*, 2006), more specifically, unmethylated bacterial CpG DNA. The sequence of DNA found in CpG DNA is taken up by cells of the body, specifically those of the immune system. Following its absorption, it makes its way to the nucleus where it enters and binds to intracellular proteins. The binding of CpG DNA to the proteins results in the phosphorylation of transcription factors needed to produce various cytokines for that particular cell. With CpG DNA as an adjuvant, the immune response involving Th1 cells becomes stimulated in a stronger fashion than the pedestrian Freund's complete adjuvant (Hartmann *et al*, 1999).

Immune System Response:

Innate Immunity:

For *Coccidioides immitis*, the innate immunity is comprised of phagocytic cells engulfing the pathogen, and secreting cytokines for the T helper cell aid. Monocytes and macrophages are responsible for engulfing *C. immitis*, however, this alone constitutes less than one percent of the cells that are eliminated. Their role transcends to producing the cytokine IL-12. IL-12 is responsible for two important factors leading to immunity against *C. immitis*: stimulating Natural Killer cells, and proliferating T cells into Th1 cells. Once stimulated the NK cells go on to produce Interferon Gamma. IFN-gamma leads to stimulating macrophages and helps them express MHC molecules on their cell surface. The other main component involved in the innate immune response to *C. immitis* is the dendritic cells. These phagocytize the pathogen and releases cytokines as well. However, dendritic cells release IL-6, which is a T- and B-cell stimulator. The dendritic cells also express the CD86 receptor molecule which once bound, stimulates the Th1. These combined factors lead to the activation of the adaptive immune response.

Acquired Immunity:

After the innate immune response is stimulated, the acquired or adaptive immunity follows. The main component in this phase for *Coccidioides immitis* is the T cells, specifically the Th1 cells. Once activated by the various cytokines produced from the innate immunity; like IL-12, IL-6, TNF-alpha, Th1 cells bind to receptors on the macrophages which have engulfed the fungus and activate them to inhibit growth of *C. immitis*. T-cell binding promotes phagosome-lysosome fusion, which serves as an impediment to the growth of pathogen. Research shows that more macrophages have phagosome-lysosome fusion if grown in the presence of Th1 cells. Furthermore, phagosome-lysosome activity is reduced once anti-IFN-gamma is introduced, thus inhibiting the stimulation of macrophages by cytokine IFN-gamma.

Humeral Immunity is not a huge effector in protecting the body from *C. immitis*; however, elevated antibody levels are still produced; especially IgG, IgE, and IgA, in instances where there is a chronic and progressive incidence of the disease. After polyclonal B cells come in contact with the *C. immitis* antigen, somatic recombination and isotype switching occurs to induce the different isotypes needed in the immune system. An increasing IgG level appears to be more involved with a disseminated *Cocci* infection. With a pulmonary infection, B cells produce more IgA since the infection is at a mucosal surface. When there is a Th2 cell response, it is commonly to see an increase in serum IgE. On the other hand, because IgE generally protects against most allergens, these levels drop after clinical signs subside (Cox and Magee, 2004).

Behind the Vaccine:

One of the main reasons Ag2/PRA was chosen as a vaccine candidate was because it elicited a strong Th1 response with minimal side effects. Through various testing stages of different forms of the antigen, IFN-gamma production increased in the body, along with an abundant T-cell response. The greater response the vaccine induces on Th1 cells and IFN-gamma production, the more macrophages there are that have active phagosome-lysosome fusion, which leads to further inhibition of fungus (Cox and Magee, 2004).

As far as the adjuvant, CpG DNA has a variety of effects to help aid in the immune response against Valley Fever. In terms of T- and B-cells, CpG DNA sends a great number, nearly 95% of B cells, into activation; and already active T-cells are co-stimulated to proliferate and secrete a greater number of cytokines. Furthermore, CpG DNA causes those activated B-cells to secrete IL-6, which further activates more B-cells and T-cells. In terms of phagocytic cells, CpG DNA has two important roles. The first major role of CpG DNA is IL-12 production, in large amounts. As stated, IL-12 stimulates both NK cells and T cell differentiation into Th1 cells. However, it also promotes early development of cytokines into IFN-gamma, whose roles both include increasing phagosome-lysosome fusion in macrophages. The other role of CpG DNA in monocytes, macrophages, and dendritic cells is a small amount of IL-6 and TNF-alpha production (Hartmann *et al.*, 1999). IL-6 stimulates T-cells and B-cells to proliferate into needed cell types, and TNF-alpha activates neutrophils, macrophages and promotes T- and B-cell differentiation. However, TNF-alpha is a main component of host-mediated pulmonary destruction (Cox and Magee, 2004). Even a small amount of TNF-alpha secretion due to the adjuvant will help decrease chances of pulmonary damage due to

host-mediated immune response, especially with an intranasal injection and the main infection of Valley Fever occurring in the lungs. With NK cells, CpG DNA causes an increase in their lytic activity towards the pathogen, causing them to release perforins and granzymes. CpG DNA also stimulates the secretion of IFN-gamma in NK cells, which, again helps activate macrophages with engulfed pathogen (Hartmann *et al*, 1999).

Immunity Assay:

Vaccine Tests:

Coccidioides immitis is a common fungus found in the soil of the southwest. This fungus is more commonly found in dogs due to the fact that dogs tend to walk with their nose to the ground. For this reason, dogs will be used as the main subject in testing the Ag-2/PRA DNA vaccine for *C. immitis*. *C. immitis* forms spores that are inhaled and infect the respiratory tract, more specifically the lungs. Since this fungus causes a respiratory infection, the Ag-2/PRA vaccine will be given intranasally to allow for a quicker immune response. All dogs will be tested for their B cell response, or humoral immunity, through an ELISA test as well as T cell response, or cellular immunity, through flow cytometry.

Humoral Immunity:

In order to test the potency of the Ag-2/PRA vaccine's B cell response, the amount of antibodies produced will be measured by the Enzyme-Linked ImmunoSorbent Assay (ELISA); levels of isotypes Immunoglobulin A, G, and M will be tested. IgA, whose primary function is antigen neutralization, is made more than any other antibody and is most commonly found underlying mucosal surfaces. Since *C. immitis* is a respiratory infection, the presence of IgA should be large; however the main focus of the test will be IgG and IgM. IgM is the first antibody in the immune response whose primary job is the activation of complement. IgG is the most abundant antibody and is responsible for memory antibodies for the secondary immune response. Because IgG is a memory antibody, a large amount should be present in the blood after a few weeks if immunity to Ag-2/PRA is stimulated. The quantity of IgG immediately after inoculation is expected to be relatively low since the memory cells will not be produced right away. Another aspect of IgG is that it has good flexibility of its Fab region allowing for high affinity binding to a variety of epitopes.

An indirect ELISA test will be done to test for the presence of these different antibodies. IgG and IgM can be isolated from a blood serum and IgA can be isolated from a sputum secretion. The first step of conducting an ELISA test is to fill a microtiter plate with the Ag-2/PRA antigen and allow it to incubate. After incubation the microtiter plate is left out for 30-60 minutes to allow the Ag-2/PRA antigen to bind to the plastic walls. Following this, the microtiter plate is rinsed to eliminate any excess antigen. Consequently, the dog's serum will be added; if the antibodies specific for Ag-2/PRA antigen are present, they will bind the antigen on the plate wall. After an additional rinse,

anti-IgG or anti-IgM will be added to bind the Fc region of the specific antibody. The Fc region of anti-IgG or anti-IgM is covalently linked with an enzyme, therefore when the chromogenic substrate is added the enzyme will convert the colorless substrate to a colored product. Using a spectrophotometer the wavelength of the colored product can be measured to determine the amount of specific antibody present within each dog's serum. Theoretically, the dog with the higher titer has more antibodies and a stronger immunity to the Ag-2/PRA antigen.

The vaccine will not only consist of the Ag-2/PRA antigen, but the CpG adjuvant as well, which is expected to induce a stronger response via co-stimulatory activity of B cells. Several controls will be placed in order to examine the validity of the ELISA test. Two positive controls will be run; one in which on the CpG adjuvant is present and the other containing solely the Ag-2/PRA antigen. The strength of the titer will determine whether a stronger immune response is present with CpG, Ag-2/PRA, or both. A negative control will also be run in which the adjuvant and the antigen will be omitted. From the results of the ELISA test the efficacy of this Ag-2/PRA DNA vaccine will be determined with regards to humoral immunity and B cell response.

Cellular Immunity:

To measure T cell response, specifically Th cells, flow cytometry will be used. In cellular immunity for *C. immitis*, Th cells are activated by the cytokines IL-12 and IFN-gamma. Thus, flow cytometry can be used to measure the quantity of cytokines produced by incubating and measuring cytokine producing cells. In this case, monocytes and macrophages are responsible for producing IL-12. These cells will be incubated in a drug block secretion of brefeldin A and then fixed with formalin. Formalin cross links the membrane so that cytokines are synthesized in the cell. Then using a detergent the cell membrane will be permeabilized to allow antibodies to enter and bind the cytokine IL-12. These cells will then be labeled with the fluorochrome fluorescein isothiocyanate (FITC) which will confer a greenish color. When run through a flow cytometer, a laser beam will measure the amount of fluorescence and charge these cells with an electricity. The electrical signal given off by the cell allows the cytometer to separate the cells as well as record them on a computer program to be analyzed. This plot will show the amount of actively producing IL-12 cells there are, which then will correspond to the amount of activated Th cells.

Another method of measuring Th response is via flow cytometry which tests the level of activated macrophages. Macrophages are very effective professional antigen presenting cells that express the co-stimulatory molecule B7, which is a ligand that binds CD28 leading to T cell activation. Therefore, in order to measure the strength of the T cell response, flow cytometry will be used to measure activated macrophages. The markers for activated macrophages are CD80 and CD86 which correspond to B7.1 and B7.2, respectively. These markers will be labeled with the fluorochrome FITC and then run through the cytometer. The electric plot produced will show the amount of activated macrophages present in the serum.

These two tests should determine the efficacy of the vaccine, however as reassurance an ELISA test can be run on the two main cytokines produced IFN-gamma and IL-12. This test may not be as valid due to the fact that the CpG adjuvant has been

known to increase the levels of these cytokines. Other cytokines that are commonly released in the immune response to Ag-2/PRA is IL-6 and TNF-alpha. Dendritic cells release IL-6 which stimulates a T and B cell response and TNF-alpha stimulates neutrophils. These cytokines may also be measured in an ELISA test, however they should be of low quantity since they are not the main cytokines in the response against Ag-2/PRA antigen.

All these tests will be run on all the dogs before any vaccine is administered to check the levels of antibodies and cytokines already present. Then, following the administration of the vaccine, the ELISA and flow cytometry will be performed again at a 2-week interval and after a month. If there is not a sufficient increase in immunity to the Ag-2/PRA antigen after a month period, then a booster may be given to strengthen the production of antibodies. All dog subjects inoculated with the vaccine should have a strong immune response if ever presented with *Coccidioides immitis*.

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