

Literature Review

Coccidioidomycosis

Coccidioidomycosis, better known as Valley Fever, is a soil-borne fungal disease caused by sub-species of the genus *Coccidioides*. Because of its ability to grow throughout the top soil of the American desert and spread via disruption, Valley Fever is endemic to most of the southwestern United States. Its initial discovery in the San Joaquin Valley of California, led to its naming: the “San Joaquin Valley Fever”. The disease is also known as the “desert fever” or “desert rheumatism” (VF center., 2007). However, the organism has a much larger distribution than that particular Californian Valley and causes problems as far south as Venezuela. This is due in part to the great affinity of *Coccidioides* to hot, dry climates. The organism is difficult to remove from these soils, providing year-round problems to those living in endemic areas. Valley Fever infection rates are on the rise. They are up 186% in Arizona alone from 1995, standing as the 4th most common disease in the state (Thornton, 2004).

Pathogen Structure

There are two different species of *Coccidioides*, *immitis* and *posadasii*, that are morphologically identical but have genetic and epidemiological differences (Rixford and Gilchrist, 2007). *C. immitis* is found in California’s San Joaquin valley region and *C. posadasii* is found in the desert of the southwest of the United States, Mexico, and South America. However, the two species are often found co-habiting these regions (Rixford and Gilchrist, 2007). Although these species have been shown to be genetically distinct by today’s standards, the separation of the species for *immitis* and *posadasii* and many other fungal microbes is somewhat controversial (Cox and Magee, 2004). For this paper, the two species discussed will be referred to together as their genus, *Coccidioides*.

Coccidioides is considered a dimorphic fungus (Saubolle et. al, 2007). Dimorphism is characterized by a two-stage life cycle. *Coccidioides* has saprophytic and parasitic forms. As a saprophyte *Coccidioides* is found in the environment and grows as a mycelia, producing thick-walled arthroconidia. This can be reproduced in the lab on media maintained at lower temperatures (Saubolle et. al, 2007). The arthroconidia in the saprophyte phase of the fungus are typically barrel-shaped and are 2.5 to 4 µm in width and 3 to 6 µm in length; the mycelia is composed of filamentous hyphal growths (Cox and Magee, 2004).

Arthroconidia only undergo their parasitic phase after being inhaled into the lungs. Here, each arthroconidium transforms into a spherical structure called a spherule and gradually grows into large cells containing uninucleated endospores. The spherule can hold 800-1,000 endospores that are released into the tissue of the host upon spherule rupture (Saubolle et. al, 2007). The released endospores, which are about 2 to 4 µm in diameter, repeat the cycle and grow into new spherules (Cox and Magee, 2004).

Pathogenesis

In a typical lifecycle of *Coccidioides* is dimorphic as described previously. The thick-walled arthroconidia (enterothallic arthroconidia) act as infectious propagules when soil disturbance aerosolizes the spores. The spores, which are only 2-4 micrometers across, are small enough to reach the alveoli of the lungs. This is where the organism

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transforms from a saprophyte into a parasite. Once in the lungs spores convert into spherules. This transformation begins with isotropic growth, which can be seen by a swelling and rounding of the cells which then undergo nuclear divisions and segmentation (Cox and Magee, 2004). During this time, there is an arrest of apical growth and progressive deputation of the hyphae; which is followed by a condensation of the cytoplasm in some of the hyphal compartments, autolysis of the adjacent cells and synthesis of a new inner wall layer. The spherule outer wall glycoprotein (SOWgp) has repeating proline and aspartic acid-rich motifs that serve as adhesins (Hung et. al, 2002). The spherules then grow in size to about 30 to 60 μm until they start to divide internally by invagination and formation of cleavage furrows (Cox and Magee, 2004). This results in the production of many uninucleated endospores which propagate the life cycle.

During infection, hosts are exposed to immature, mature, and rupturing spherules as well as endospores at the same time. In vivo, the spores are taken in through inhalation but can be administered cutaneously in clinical trials. It has been discovered that the growth of endospores into new spherules is influenced by the presence of phagocytic cells and an increase in CO_2 (Saubolle et. al, 2007). The symptoms for this disease include fatigue, cough, chest pain, fever, rash, headache, and joint aches. However, the clinical signs are unapparent over 60% of the time and are mild or moderate 30% of the time. Patients only suffer serious complications in 5-10% of cases, with less than 1% patient mortality.

Epidemiology

Coccidioides is endemic to the western hemisphere; with its highest prevalence in the southwestern United States and border regions of northern Mexico. Areas of lower endemicity include localized regions of Central and South America, such as Venezuela and Brazil (Cox and Magee 2004, Kirkland et. al. 1997). Specifically in the United States, endemicity centers on south central portions of Arizona, the San Joaquin Valley of California, New Mexico and southwestern Texas (Cox and Magee 2004, Kirkland et. al. 1997). It is estimated that in the southwestern United States there are 100,000 new infections every year (Kolivras, 2004). This distribution corresponds to the Lower Sonoran Life Zone, which is typified by arid to semi-arid desert (Cox and Magee 2004). The fungus is often found unevenly spread 4 to 12 inches below ground level (Kirkland et. al. 1997).

Yearly incidence of *Coccidioides* infections varies from season to season with the highest reported rates during the dry late summer and early fall immediately following a rainy season (Cox and Magee 2004, Kirkland et. al. 1997, Kolivras 2004). Since the organism propagates as mycelia in moist environments, the timing and amount of precipitation in endemic regions play significant factors in yearly disease incidence (Cox and Magee 2004, Kolivras 2004). During dry periods, arthroconidia form and are aerosolized through any manner of dust dispersal: wind, natural disaster, or any other soil disturbance. Disease incidence is minimized during the rainy seasons, as dust levels are reduced (Kolivras, 2004).

Since *Coccidioides* causes a primary respiratory tract infection in humans, the most critical risk factor for infection is exposure to dust via inhalation. Person-to-person transmission effectively does not occur in all but the most outstanding situations (Kirkland et. al. 1997).

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Frequent dust storms in these regions are often followed by disease outbreaks. A particular outbreak in 1977 occurred in the San Francisco Bay area following a severe dust storm that carried particles from the San Joaquin Valley. It caused hundreds of cases. However, the largest outbreak of *Coccidioides* in the United States occurred during World War II when multiple airfields were built in the San Joaquin Valley. New infection rates among military personnel soared from 8% to 25% per year and *Coccidioidomycosis* was the most common hospitalization cause at numerous airbases in the desert southwest (Kirkland et. al. 1997). This indicates that any form of soil disturbance can cause aerosolization of *Coccidioides* spores and spread of disease propagules.

Risk Populations

There are certain groups that are more susceptible to infection, as well as groups that have a greater chance of disease progression to fully disseminated *Coccidioidomycosis*. The most susceptible groups, by far, are pregnant women in their third trimester, the immunocompromised, infants, and young children. Concerning gender and race, males are more likely to contract the infection as well as African American and Asian populations. This is simply due to different immune structures in each person's body. Population risk due to exposure and occupation includes construction workers, farmers, archeologists, and persons partaking in recreational activities in endemic areas, such as mountain biking or four wheeling (VF center, 2007). Also, those not raised in endemic areas that visit these regions have higher chance of exposure and infection due to no previous contact with the organism and no developed immune response.

Animals are also at risk, as they are commonly found digging or rutting into infected soil, increasing their likelihood of exposure and infection by spores. The animals that Valley Fever has been noted in include dogs, cats, horses, llamas, monkeys, zoo animals, marine mammals, and wildlife in endemic areas. *Coccidioides*' infectious ability is not limited to these populations and species as it has been able to infect all living animals it has been tested on (VF Center, 2007).

Also, patients receiving organ donations from endemic areas may be at risk, as there is the chance disseminated *Coccidioides* has spread to the donor's organ. It has been found that approximately 2% of organ donors from Arizona have transmitted an asymptomatic form of Valley Fever to their recipients. Upon retrieval, the recipient requires antifungal prophylaxis with Fluconazole, which keeps the fungus asymptomatic in the recipient (Schienzer, 2006).

Current Treatment and Previous Vaccine attempts

Since *Coccidioides* is a Eukaryotic organism, Valley Fever is a difficult infection to treat and remove from the body. Currently, there are a few known treatments for this infection that bring the antibody titer down to normal levels. The main type of treatment is with antifungal medication; such as Ketoconazole, Fluconazole, Intraconazole, and even Amphotericin B. The different medications are normally decided upon by the severity of infection, with Amphotericin B used to combat the worst and provide the quickest recovery from the problematic stages. There is a new antifungal, known as Voriconazole that is currently being tested and is not approved by the FDA. The others,

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however, are approved and administered commonly (Galvani, 2005). Treating this disease becomes much more difficult when it has reached the disseminated stages and/or is infecting an immunocompromised person. Due to the altered cell – mediated immunity that comes with the infection of AIDS, Lymphoma and even organ transplant recipients with T–cell suppression therapy, individuals who become infected with *Coccidioides* must go on a rigorous treatment with IV Amphotericin B as well as long term antifungal; any of the azole medication (Menendez, 2005)

Currently there is no vaccine for Valley Fever; however there have been some attempts with mice to illicit immune responses. In one such experiment, there was a wide variety of mice used, from normal mice to ones with mutation in their Interleukin 12, interferon γ , CD4⁺, CD8⁺, and MHC II. These knockout mice were used to see exactly what portions of the body’s immune response was necessary. The mice were immunized at two occasions, two weeks apart, with an adjuvant and then infected with *Coccidioides* two weeks after the second immunization. The appearance of spores in the lungs was the determining factor in whether or not infection occurred in the mice.

The findings of the study showed that Th2 cells were essential in providing protection from Valley Fever, as well as TNF α cells. Th1 and CD8⁺ cells were not absolutely required as without them protection still occurred, but when present aided in a greater amount of protection for the individual. The main finding was that “vaccine efforts must focus on MHC–II restricted responses” (Molecular... , 2006). Absence of either IFN γ or IL – 12 would cause ineffective immunization. This study’s suggestion was formulated to be this: “activation of macrophages by IFN γ produced by class II – restricted T cells in an IL – 12 rich milieu may be a crucial step in controlling infection” (Molecular... , 2006).

This work has not been done in any human setting. The only attempt with human vaccination came following an animal test. The animals were successfully vaccinated with formalin – killed spherules. This, however, did not occur in the human test in preventing any infection. The reason is still unknown, but is considered to be due to the need for a T_{h1} response to come from a protein based vaccine, which is a medically impossible in human subjects to date (Molecular... , 2006)

Cost-Effectiveness and Importance

The development of an effective vaccine has both clinical and economic importance to those residing in endemic areas. It has been determined using a decision model that it is most cost-effective to vaccinate children in these areas, with an estimated savings of 1.9 quality-adjusted life days and \$33 per person; as compared to the cost of antifungal treatment, which ranges between \$100-\$250 dollars every three months (Barnato, 2001). Vaccination of adults would prevent disseminated Coccidioidomycosis, but have a net increase of health costs of \$62,000 per quality adjusted life year over no vaccination (Barnato, 2001). It is for this reason that we propose our vaccine be mandatory for young children, especially since lifelong immunity would be induced after exposure, protecting the vaccinated into adulthood.

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Vaccine

Description of Vaccine

Live attenuated vaccines contain weakened forms of the pathogenic organism. They normally are made by culturing the organism in media or in cells to an end where a specific virulence factor is reduced or the entire organism loses pathogenicity as a whole. One of the good characteristics to a live attenuated vaccine is that it is likely to introduce one or more specific mutations so that the host's immune system is exposed to many serotypes or differences in the organism. With *Coccidioides*, there really are no observed mutations as it evolves at a much slower rate from generation to generation than many other pathogens. This means that immunization with one *Coccidioides* strain will provide protection against respiratory challenge from a phenotypically or genotypically different strain (Pappagianis, 1999).

We chose this type of vaccine for *Coccidioides* to illicit an immune response that induces the creation of memory cells. Once a person has recovered from a benign or asymptomatic infection with this particular organism, they are typically resistant to re-infection (Cox and Magee, 2004). This is strong evidence to support that memory B and T cells are developed with one infection with *Coccidioides*.

To increase effectiveness from previous vaccine attempts, this vaccine will only contain high concentration of immature spherules. Because a mature spherule can grow to be 50 to 100 μm , it often escapes an attack by the macrophages (Kirkland et. al, 2005). Our vaccine would require genetic modification of the *Coccidioides* spherules to basically "freeze" them in their maturation state at a size that would be conducive to ingestion by the macrophages. This would be done by inducing a point-mutation in the developmental region of *Coccidioides* DNA in order to create a stop codon. This stop codon would then create a non-functioning amino acid and prevent the organism from developing any farther than the spherule stage.

The antigen that would be inducing an antibody response in our vaccine would be a surface protein on the spherule. The protein of interest is called antigen-2/proline rich antigen (Ag2/PRA) is located in the wall of the spherule and has been found to have no homology with any mammalian protein (Kirkland et. al, 2005). This is important in that we would not want the body to react to self-proteins when given this vaccine. In addition to the genetic modification that would prevent full maturation of our spherules, expression of the Ag2/PRA would also be increased dramatically; ensuring that the spherules are readily recognized by macrophages and coated by antibodies. This increases the immune response to the invasive organism counteracting the fact that it is unable to reproduce, and ergo have higher concentrations of binding antigen. Without this, the vaccination would prove ineffective, as the organisms introduced to the body would likely have too small of a potential immune response to illicit memory cell formation.

Route and Timing

The vaccine would be administered twice to young children. The first vaccine would be intranasal and would be given at 6 months old. The intranasal vaccination will include a high percentage per volume of immature spherules in a mild saline solution. This would elicit a mucosal immune response and the antibody IgA. The next vaccine in

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the series would be given 3 months later at 9 months of age. The will be given sub-cutaneously and will elicit IgG and IgM antibodies as well as reinforce the cellular and humoral responses created by the initial infection. The sub-cutaneous injection will have a higher concentration of *Coccidioides* and will also be suspended in a mild saline solution. This booster will ensure that proper levels of memory cells are created to defend the individual throughout life.

Immune response to vaccine

Injection with the live-attenuated, genetically modified vaccine for *Coccidioides* vaccine will elicit a full immune response from the innate, humoral and cellular immune response. Because the vaccine is administered both intranasally and cutaneously, it will cause slightly different responses by the immune system.

The first infection with *Coccidioides* will be administered intranasally to persons that are six months old. When the antigen enters, it will travel from the tissue to the blood where it will be picked up by M cells (Parham, 2004). These M cells will send it to the mucosal tissue for phagocytosis. This will first cause an innate immune response followed by an initial adaptive immune response a few days later. Once the adaptive immune response finishes fighting the infection it will be adequately prepared for a second infection.

The second infection with the vaccine will be administered cutaneously in the arm via injection. This will be the secondary vaccine and will be administered to the same patients at nine months of age, approximately three months after the initial vaccine. Again, the innate immune response will be activated but a much stronger adaptive immune response will be present. This will strengthen the Th1 cells and will help the body to more easily and quickly rid itself of the infection.

As mentioned before, both the innate and the adaptive immune response will occur to the infection with *Coccidioides*. In the innate immune response, polymorphonuclear leukocytes (PMNL) ingest the spores that cause a respiratory burst that releases cationic peptides (defensin). However, mature spherules are often 60-100 micrometers in size while the PMNLs are only 12 micrometers. Thus, these only kill their targets 20% of the time (Cox and Magee 2004, Parham 2005).

Natural killer cells (NK cells) and dendritic cells (DCs) are also activated by the innate immunity. NK cells are activated by IL-12 which helps initiate cytokine production. They also produce IFN-gamma which activates macrophages and IFN-alpha which is effective in development of cytotoxic effector functions. NK cells recognize altered MHC on infected cells and use IFN-gamma and TNF-alpha to kill the infected cells. This helps to significantly reduce fungal viability. DCs take up the endospores and travel to the lymphoid organs to act as antigen-presenting cells (APCs). While in the lymphoid organs, they mature and lose endocytotic activity (Cox and Magee 2004, Parham 2005).

Macrophages and monocytes also play a role in the innate immune response. Macrophages engulf the spores and take them into intracellular vesicle. Macrophages release oxygen radicals and nitric oxide (NO) that are toxic to pathogens and their host cells. This is important because it enables macrophages to attack molecules that are too large to phagocytize. Macrophages also produce CCL2 which attract monocytes, leukocytes, NK cells, T cells, dendritic cells, and cause basophils to release histamine.

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Monocytes are recruited by CCL2 and cause NO and TNF production. Monocytes are bound by proteins and carbohydrates from lysed macrophages. However, *Coccidioides* lead to inhibition of phagosome-lysosome fusion and consequently, less than 1% of phagocytized cells are killed (Cox and Magee 2004, Parham 2005).

Complement is also activated by *Coccidioides*. Complement provides a physical barrier by means of platelets and clotting factors. Moreover, it promotes tissue repair which is initiated by response of macrophages to pathogens. On top of this, complement increases vascular diameter which cause heat, swelling, and redness to occur (Cox and Magee 2004, Parham 2005). The endothelial cells lining the blood vessel walls also express adhesion which binds leukocytes that migrate into the tissue and helps slow down blood flow. Ultimately, it causes the entry of plasma into the infected tissues and help drain the lymph fluids.

Coccidioides also causes cellular immune response. T cells and B cells play the ultimate role in the cellular immune response. Activating DCs lead to both CCL3 and CXCL8 secretions and maturation of DCs into APCs. To do this, the DCs up-regulate MHC I and II molecules as well as CD83, CD40, CD58, CD80, CD86, CCR7 and CXCR5 molecules. CCR7 and CXCR5 are important because they interact with the T and B cells (Cox and Magee 2004, Parham 2005).

Helper T cells (Th1) recognize degraded proteins on MHC of DCs. Th1 produce IL-2 which activates T and B cells. Th1 cells also produce IFN-gamma and TNF-beta which cause phagocytosis. They also bind to B cells that are presenting *Coccidioides* on MHC and activate them. Th1 cells activate macrophages that have engulfed *Coccidioides* using IFN-gamma and CD40 ligand (Parham 2005). They can also use Fas ligand or TNF-beta to kill chronically infected macrophages that are releasing the *Coccidioides*. Th1 cells also use TNF-alpha and TNF-beta to activate endothelium to cause macrophage adhesion and to exit form the blood vessel at the site of the infection. On top of this, Th1 cells use CXCL2 and CCL2 to guide macrophages to the site of infection (Cox and Magee 2004).

B cells have antigen-specific receptor Ig and bind to antigen *Coccidioides*. Both B cells and DCs present peptides to Th1 cells via MHC and B cells become activated. Upon activation, B cells become plasma cells that produce specific Ig antibodies, in particular IgA and IgG.

Macrophages, DCs, CD4+ and CD8+ T cells, and B cells all produce TNF-alpha. This helps to activate neutrophils, enhances cytolytic activity of macrophages, augments NK cells activity and promotes the proliferation of T and B cells. Also, immune CD69 and CD3 lymph increase which leads to increased IFN-gamma, IL-2, and TNF-alpha (Cox 2004 and Parham 2005).

Humoral activity is also initiated by *Coccidioides*. Chronic Coccidioidomycosis has been associated with polyclonal B-lymphocyte activation. This is supported by the increase levels of IgG and IgA. The internasal injection will illicit a stronger IgA response. Elevated IgA will help with neutralization and activation of the complement system. This is supported by the fact that approximately 20% of all patients with chronic pulmonary disease have elevated levels of IgA. On the other hand, the cutaneous injection of the *Coccidioides* will cause more IgG antibody production. IgG also helps with neutralization and activation of the complement system as well as with sensitization

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of the pathogen for killing by NK cells. In fact, IgG has been correlated with multi-focal disease involvement (Cox and Magee 2004).

Anticipated Problems and Safety measurements

Because the initial vaccine is intranasal, there is a chance of recipients having a small respiratory response to the *Coccidioides* in the form of a mild respiratory infection. This however, will be dealt with in the clinical trial and the anticipated percentage of testers who will develop minor symptoms will not be considered negative. Those suffering from secondary infections or more severe respiratory responses will be treated on a case by case basis. This is not expected to occur in more than 1% of the population tested. Intranasal administration may be uncomfortable for the infant and cause mild irritation of the nasal area for a short period of time.

Primary problems with the sub-cutaneous booster will be mild discomfort in the area of vaccination, in regards to the needle prick, and a small inflammatory response at the site of administration. This is due to the aggregation of macrophages attempting to ingest the spherules that were administered. This discomfort, however, will fade rapidly and is not considered a strong negative side effect.

The most significant problem for this vaccination regimen will be ensuring that proper genetic recombination is conducted to produce the necessary immature spherules. Testing will have to be vigorous with a low error margin as it is possible a super strain could be produced that is more virulent than previous incarnations of *Coccidioides*. Tissue testing will have to be conducted on each batch of vaccine produced, to reduce this risk and ensure that the spherules do not mature and produce endospores.

If any form of disseminated infection occurs from either the initial intranasal or sub-cutaneous vaccinations, testing will be suspended immediately for the patient in question until the cause is determined. If this occurs in greater than 1% of the test population, the vaccination program will be shut down for reevaluation.

Immunological assessment

In the past, the primary test for prior *Coccidioides* infection has been the application of one of two dermal skin tests: Coccidioidin or Spherulin©. However, it has been shown that neither agent has 100% reactivity with patients whom have had previous infections (Dodge et. al., 1985). For this reason, we will not be using skin testing to verify positive reactivity to our vaccine, as it provides inconsistent results and no information on T or B cell memory testing or no antibody presence.

Antibody Testing

There are two different tests that can show an antibody reaction to *Coccidioides*. The first one is through an ELISA test, where IgG and IgM antibodies will be measured. This test is preformed 1 week post vaccination for the 9 month old child. The child will have its blood drawn and spun down to serum. The ELISA assay itself will be coated in pure *Coccidioides* antigen Ag2/PRA and the serum sample will be added to these well plates. If antibody is present in that child's blood, it will cause a color change in the well once a substrate has been added. This will show the amount of immunity prevalent in the child's blood stream. The isotypes the ELISA will be noting are the IgM and IgG. These isotypes will be used in order to show how rapidly the vaccine works in producing the

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isotypes, and whether or not the initial intranasal vaccine provided enough support to elicit a rapid secondary response with a larger amount of IgG over IgM. This type of antibody test was decided because of the type of vaccinations that are administered. Because the infant should receive IgG and IgM from its mother via the placenta for the *Coccidioides* antigens from her own vaccinations, the first intranasal should primarily elicit an IgA response from interaction with the mucosa. The second SQ vaccination and blood test are to show that the baby has moved away from the mother's isotype production and is in fact producing IgG and IgM for *Coccidioides* on their own.

T-cell testing

Testing for T cell memory will be a two-fold process. Cell samples from our test population will be collected at an alternating day intervals following primary and secondary vaccination for at a minimum of 2 weeks, or until satisfactory results are obtained. For both the primary and secondary vaccinations, blood samples will be taken and spun down to serum. This serum will be used in the testing for T cell memory.

First, samples will be analyzed with antigen specific flow cytometry equipped with fluorescence-activated cell sorter (FACS). In doing this, we will be able to separate out CD4 and CD8 T-cells that are specific to the antigen processed from encountering *Coccidioides*. Since T-cells are unable to bind antigen directly for fluorescence we will need to incorporate Tetramer MHC-peptides specific for *Coccidioides*; one for MHC class I on the CD8 cells and another for MHC class II on the CD4 cells. Testing for each cell type will be run on separate trials to minimize cross reactivity and interference, and will be run with a negative nonspecific peptide control. Results can then be run against total T-cell counts for efficacy of the vaccine's ability to produce effector T cells, and ergo memory T cells.

Once separation is complete, T helper effector testing will be conducted for Th2 cells. This is conducted by testing for the presence of the specific cytokines Interleukin 21 (IL-21) which is specific to Th2 cells and stimulates the proliferation of B, T, and NK cells. In a normal immune response to *Coccidioides*, Th2 cells specific to Ag2/PRA antigen normally release IL-21 when the cell encounters and binds antigen. This will be done using a similar flow cytometry test as described above. Collected CD4 cells will be placed in media with *Coccidioides* antigen Ag2/PRA and an inhibitory drug that will block cellular release of the cytokine IL-21. Once these cells are allowed to incubate and build up a significant IL-21 supply they will be treated with formalin that fixes the cytokine supply inside the cell via cross-linkage. The cells are then treated with detergent to make the cell wall permeable in order that fluorescent antibodies specific to the IL-21 may be introduced into the cell and bind. These cells will then be run through a flow cytometer again reading for fluorescence produced by the IL-21 bound antibodies; quantifying the amount of Th2 cells in the system.

Cytotoxic effector functionality will be tested using a 51-Cr release assay. This will act as a supplement to the ELISA and Th2 testing which are of primary importance in determining proper immune development for a vaccine to *Coccidioides*. This test essentially examines CD8 T-cell function by determining if they kill target cells. Live cells will take up, but not spontaneously release, radioactively labeled sodium chromate. When these cells are killed the chromate is released and its presence can be observed in the fluid mixture of target cells and T cells.

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Anticipated Problems with Immune Testing

ELISA testing can sometimes give false positives or negatives which may interfere with immune testing and cause subsequent problems in determining efficacy of the vaccine. Since this test is prone to these errors, testing will be repeated to verify the results.

Problems with flow cytometry testing may include ineffective binding of antibody to IL-21, ineffective production of cytokine IL-21 *in vitro*, and low resolution on cytokine fluorescence. Ineffective antibody binding would likely be the result of poor cell wall permeation. The antibodies simply would not be able to enter the cells and bind, ergo they would wash off and the cells would not fluoresce. Ineffective production of IL-21 may happen if the cells used are not specific for the Ag/PR2 antigen or if some kind of interference occurs that was not anticipated between the *Coccidioides* antigen and the cytokine resulting in insufficient IL-21 production. Low resolution on fluorescence is tied to this previous problem, as a lack of IL-21 would cause minimal if any fluorescence to be read by the cytometer. This could also occur if the Th2 cells do not produce sufficient IL-21 in any situation for it to be read. In this case, another cytokine would be tested, IL-4, -5, or -10, however, this is not preferable as IL-21 is specific to Th2 cells whereas the others can be produced by other T-cells, in certain situations.

Criterion for a Successful Trial

To determine whether or not our vaccine is effective at providing immunity to *Coccidioides*, we expect to have a positive immune response in 85% or greater of our test population. A positive immune response to the vaccine will be defined as when IgA and IgG antibodies are elevated following a vaccination as well as elevated Th2 cell counts with appropriate cytokine functionality.

Population size will be determined during the trial, but must be significant in size to minimize probability errors. Statistical testing will be used to analyze acceptable immune responses in patients in comparison to our 85% cutoff with a α value of 0.05, giving us a 95% certainty of the obtained results.

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