Subunit Vaccine for Immunosuppressed People with *Coccidioides immitis*

Literature Review:

Coccidioidomycosis commonly known as Valley Fever is a widespread disease common throughout the US southwest, with the most endemic regions being across southern California, Arizona, New Mexico , Texas and the desert areas of Northern Mexico. *Coccidioides immitis,* the fungus that causes *Coccidioidomycosis*, lives in the soils of these southwest regions where there is low rainfall, high summer temperatures and moderate winter temperatures. Small spores become airborne when the soil is disturbed. Spores can survive as deep as 20 cm under the soil. Theses small spores known as arthroconidia spores are inhaled where they attach to their primary site of infection, the respiratory tract and lungs. Within the lungs the spores change into larger multicellular structures called spherules. Spores can travel through the bloodstream to other parts of the body, such as the brain and central nervous system where they can germinate and grow causing severe complications.

Once spores are inhaled they create lesion on the lungs and are capable of spreading to bones, joints, and soft tissues. Arthroconidia spores have a specific life cycle within living organisms and are able to self-replicate within the organism. (Jinadu 1995)

There are three distinct stages of infection: acute, chronic and disseminated. Acute valley fever form is often mild, with few if any symptoms, symptoms can include fever, cough, chest pain, fatigue or headaches. Chronic valley fever can stay present as many as twenty years after the initial infection. The chronic form is most common in people with diabetes or weakened immune systems. Disseminated valley fever is the most serious form of the disease, because the infection spreads beyond the lungs to other parts of the body, most often the skin, bones, liver, brain and heart. With the disseminated form painful lesions in the skull, spine or other bones can be present. Although there are three stages, each individual's infection is unique, meaning that all three stages are possible, but not necessarily developed. Sixty percent of infections are asymptomatic, while forty percent of cases develop symptomatic pneumonia, rash or desert rheumatism. One hundred thousand cases are developed and documented each year throughout the endemic regions, making this a very important disease to research and eventually find a vaccine for. Coccidioides is the most virulent of the fungal pathogen. (*Kirkland*)

Although there are several symptoms that accompany Valley Fever, most infected people do not know then have the disease, unless confirmed by microscopic identification of the fungal spherules. Growing a culture of the *Coccidioides immitis* from body fluid or tissue specimen can be done, and also detections of antibodies against the fungus in blood serum or other body fluids. In cultures, the arthroconidia spores will not be produced until five or ten days. Valley Fever skins test (called coccidioidin or spherulin) can also be performed; to indicate prior exposure to the fungus, since reactivity is lifelong it's not particularly helpful in diagnosis for current infections. In most States the skin test is not available. A routine chest X-ray can also be done to detect Valley Fever cavities, in individuals that do not have any symptoms. If cavities are present, surgery can be performed to remove the cavities in the lungs, and surgical drainages of Valley Fever lesions in bones or joints.

Coccidioidmycosis is not a new disease; it has been around for about two hundred years. Alejandro Posadas discovered the disease in 1891 in a hospital in Buenos Aires, Argentina, when one of his patients developed it; he was able to study the progression of the disease over the course of seven years. Although it has been around its not until recently that cases have increased dramatically. Within the past twenty years there has been an increase in cases in Arizona and California, theses outbreaks are estimated to cost millions in direct medical expenses.

Valley Fever affects many other species besides humans, including dogs, horses, cattle sheep and other domesticated animals. Due to the fact that this is a fungus, it is very seasonal. Depending greatly on seasonal rainfall in the spring and flourishing in late fall and winter. Other than respiratory routes of infection, transmission via open wounds is possible. *C. immitis* is not contagious and will not spread person to person.

Even though there is no known vaccine for Valley Fever there are a few treatments available for patients to take depending on what stage of *C. immitis* they have acquired. Fungal drug therapy contains amphoericin B, fluconazole, or intraconazole. This therapy is conducted for several months or several years. Even if theses medications are successful in treating an individual, there are some major side effects that may occur. For example, the amphoericin B can be very toxic to immune suppressed patients. Fungus diseases are very difficult to treat, because it is very hard to find drugs that will kill the fungus without killing the human or animal host. Individuals that do not want to do treatment take the alternate route and focus on the use of herbs, vitamins, sulfur and other non- pharmaceutical drugs. However, in most cases individuals with Valley Fever recover with no treatments and will have life-long immunity.

C. immitis can affect everyone, but there are certain populations that are more susceptible to the fungal disease. Individuals who are at a higher risk are those that are immune suppressed including organ donors, AIDS/HIV patients, diabetics, population of individuals over the age of sixty, women in their third trimester of pregnancy. Those of African American, Filipino or Hispanic descent are more likely to develop the disease compared to other nationalities. Filipinos are 176 times more likely to get disseminated form of the disease then Caucasians. Indians and Hispanics have a higher mortality rate of the disease compared to Caucasians. Men are more likely to get it then women and individuals whose occupation is construction, agriculture work, work involving disturbance of desert soil and archeology are at higher risk to develop the disease, because of the greater risk of being expose to arthroconidia spores. (Jinadu 1995)

There are a hundred thirty thousand *Coccidioides Immitis* cases recorded a year. Fifty percent of those individuals with *C. immitis* show symptoms of meningitis, pneumonia, and flu like symptoms, which could end up fatal.

By developing a vaccine for Valley Fever, it will help to protect humans from contracting *C. immitis*, especially those individuals living in the southwest region of the United States. Since, our own human immune system creates its own immune responses mechanism; it will be easier to produce a beneficial vaccine. (Hughes 1998)

Description of Vaccine

Vaccine Type:

The vaccine that seems most applicable to our pathogen would be a recombinant subunit vaccine. The vaccine will target antigens of *Coccidioides Immitis* and help induce immunity. Specific antigens that the vaccine will target are Ag2 and PRA; both are antigens that are part of the cell wall. (Shubitz, 2006) This vaccine will require an adjuvant, CPG OGN, which will help induce a T-cell mediated immunity response to the pathogen and the antigens. (Tarcha, 2006)

Subunit vaccines have purified antigens rather then containing whole virulent organisms. The Subunit vaccine will not carry the live pathogen or the whole species, which means it is safe to give to immunosuppressed people. This is beneficial for our pathogen, since immunosuppressed individuals are the main subject population of our vaccine. Also, subunit vaccines are less likely to induce unfavorable immune reactions that may cause side effects.

The Subunit vaccine will be injected intramuscularly and the adjuvant will also be injected intramuscularly for a total of 4 injections. The vaccine and adjuvant will be used in a span of twelve months, with the adjuvant being injected initially with the vaccine and then alone every threes months from previous injections.

Type of immunity:

Since this is a fungal disease, the vaccine that will be created will induce a T-cell mediated and humoral immunity. As most research of the infection has indicated, T-cell mediated immunity would be best to eliminate the infection because macrophages play a significant role in people who already have an adaptive immune response to this pathogen. (Tarcha, 2006). This vaccine was chosen because it seems to cause the least amount of risk to our test population and those who get infected in non-control environments. Recombinant subunit vaccine has few side effects and would be a good candidate to those with suppressed or compromised immunity. (Barnato, 2001) The pathogen is also relatively large, so being able to pick certain proteins to grow in yeast cells will not be too difficult. The vaccine is also cost efficient, easy to maintain and store.

Antigen type and T cell activation:

The antigens that will be tested in this vaccine are Ag2 and PRA. These antigens were chosen because people who are immune to *Coccidioides immitus* have antibodies to Ag2 and PRA. In addition, these antigens are on the cell wall, which will be more easily extracted to try and create a vaccine.

This would work because the injection of the peptides would allow the body to produce CD4T-cells or T-helper cells specifically, TH1 cells. Once injected, the foreign protein will be phagocytosed by macrophages. Macrophages will break down and process the antigen and present it on its MHC II, allowing TH1 cells to bind and recognize the foreign peptide. Once the TH1 is bound to the MHC II, it can send a signal to the macrophage to eliminate the pathogen inside of it. IFN-γ is a primary signal that will be released by TH1; this signal will help induce the macrophage to kill the antigen living inside its vesicles. Other important reasons our vaccine will induce TH1 cells are due to the different cytokines and chemokines that the TH1 cells releases including our IFN- γ, there is also Interlukin-2, which will signal more T-cell proliferation, increasing the chance of a better immune response to *C. immitus*.

In addition to our TH1 cells, activation of CD8 T –cells will also be induced by our vaccine. The activation of CD8 T-cells will also occur with dendritic cells and the co-stimulation of our already present CD4 T-cell. Infected dendritic cells can activate CD8 T-cells using B7 and IL-2 which is released by CD4 T-cells. The activated CD8 T- cell will contribute to immune response by secreting signal molecules such as the IFN- γ, which also helps in signaling macrophages. Further more, infected macrophages can signal CD8-T cells by presenting peptide on MHC I. Once bound, the cytotoxic T- cell can send out lytic granzymes, this signals the infected macrophage to begin apoptosis. (Parham, 2005) Overall, the main goal of the vaccine is to induce a T-cell mediated immunity. In turn this activation will be specific to induce CD8 T-cells and TH1 to produce the cytokine IFN- γ and IL-2. IFN- γ will signal macrophages to kill *C. immitus*, while IL-2 will help proliferate more T-cells, so more IFN- γ can be released.

B-cell activation:

B-cells are fully activated with the help of CD4 T-cells that provides the B-cell cytokines and signaling of the CD40. During the process when B-cells begin to proliferate themselves, memory cells and plasma cells are also being created. IL-2 with the help of antigen CD4 will provide a signal to B-cells to induce immunoglobulin in this case IgG. CD4 T-cells on their own can also help B-cells proliferate and signal immunoglobulin to secrete.

Adjuvant:

The adjuvant CPG OGN is also important for the vaccine to be most efficient. This adjuvant helps induce IFN-γ and IL-12 to activate macrophages, dendritic cells and natural killer cells. Although TH1 cells and CD8 T cells produce IFN –γ, the adjuvant acts like a booster

The adjuvant CPG OGN can also help induce an inflammatory response. In this case toll like receptor-9 (TLR-9) will be turned on and provide a cascade event. (Roeder, 2003) TLR-9 is turned on because this receptor responds best with CPG-DNA motifs which our adjuvant contains. The CPG-DNA motif is usually characterized in bacterial DNA and not usually in humans.(Takeshita, 2001) For this reason, when CPG-ODN is injected and binds to human TLR-9, cellular immune responses can occur.(Roeder, 2004) When CPG-ODN binds with TLR-9, TLR-9 will then activate and bind to MyD88,

continuing the cascade and eventually a release of inflammatory cytokines will occur through NF-κB. The inflammation induced will allow more white blood cells to go towards the site of infection making it a better possibility to have more macrophages engulfing *C. immitus.*

Interlukin -12 (IL-12) plays a role because this cytokine is released by macrophages and activates natural killer cells (NK cells). This is important because activation of NK cells allow the cell to release more IFN-γ. According to Parham macrophage secretion of IL-12 and NK cell secretion of IFN-γ creates a positive feedback, allowing an increase of both cells within an infected tissue. (Parham, 2005)Activation of both cells will allow the vaccine to work more efficiently and in a quick manner.

In summary, the injections will allow the human body to develop CD8-Tcells, TH1 cells and B-Cells with antibody IgG and this will also create memory B and T cells if there are further infections. Which for people with great immune systems already have for *Coccidioides immitus*.

Problems with vaccine and antigen:

With any vaccine there are certain problems that might be encountered. One problem from this vaccine is that the whole pathogen is not used, but only certain proteins from it, which does not allow for a great immune response. One proposal to solve this problem is to have yearly vaccinations, similar to that of the influenza vaccine. Yearly vaccines can assure that if the first inoculation did not induce a strong response, then multiple exposures to the antigens would provide a better immune response.

Another problem with the vaccine is that the subunit proteins may not keep their native conformation and antibodies made to this subunit may not be the same as with the actual pathogen. One way to make sure this does not occur is by testing the antibodies though the ELISA test and making sure that the antibodies produced are ones that fight the pathogen. If not, then another subunit vaccine can be produce making sure that the proteins do not change.

In summary, the subunit vaccine seems to be the best method to try and induce immunity to *C. immitus*. Since the proteins are grown in yeast cells there is a less likely chance that endotoxins will be produced, making this more safe and reliable. (Subitz, 2006)

Immunity Assessment

We have chosen the subunit vaccine using Ag2/PRA (antigen 2/proline-rich antigen), with a CpG ODN adjuvant (Cox. 2004). The CpG ODN adjuvant will be administered to the subjects simultaneously with the Ag2/PRA vaccine. And an additional adjuvant booster will be administered three weeks after the first CpG ODN.

We will be looking for an increased production of interleukin 12, IFN gamma, IgG. Each of the aforementioned immune components are hallmarks of the Th1 immune response; the immune response necessary for immunity against Coccioides Immitis (C. Immitis). To accomplish this we will utilize the ELISA, ELISPOT, Cytoxicity, Flow Cytometry, and Peripheral Blood Leukocyte assays. ELISA will used primarily for the detection of IgG1, IgG2 and Interleukin 12. The ELISPOT assay will be used to detect the increased levels of INF gamma. Flow Cytometry will be used to quantify the antigen specific CD4 T-cells. The Cytoxicity assay will measure the CD8 T- cell present which contribute to the immune response by secreting signal molecules such as the IFN-gamma, and in the activation of macrophages. The peripheral blood leukocyte level assay will be used as a safeguard to ensure that our vaccine is in fact eliciting the immune response we need; the Th1 immune response.

The Ag2/PRA vaccine was chosen because of its ability to induce a Th1 IgG immune response. The adjuvant CpG ODN was chosen because of its ability to increase the production of both INF gamma, and Interleukin 12 both of which are also extremely important in the Th1 immune response. Mice that have demonstrated protective immunity against Coccioides Immitis in previous studies have increased levels of interleukin 12, IFN gamma, IgG, IgM, and MHC class 2 restricted t-cells all of which are hallmarks of the Th1 immune response.

Our study, on vaccinated patients, will take place over a period of one year. Our vaccine will be administered intranasally and subcutaneously in immuno-competent patients. Previous studies have shown that mice infected intranasally with very few parasitic spores have become terminally ill from the infection. This is due to the fact that the nasal passages would be the easiest way for the spores to reach the lungs for incubation. We will also use the subcutaneous method since that is another possible avenue for infection. We expect to see a similar pattern of infection with our human subjects.

The typical pattern for illness for Coccioides immitis is not easily distinguishable from other various respiratory tract infections (Cox 2004). This of course will present a problem for us when distinguishing between a C. Immitis infection or another common respiratory problem. Another possible problem we could face is the difference in infection rates between different ethnic groups. In similar studies the difference in ethnicity has proven to affect the rate of infection. another major problem with the vaccine could occur if the wrong immune response is activated.

Tests to be Administered

ELISA

An ELISA assay for antigen specific antibody production was utilized to detect IgG1,IgG2, and interleukin 12. A microtiter plate with purified antigen (in the wells) was allowed to sit for forty five minutes before being washed. Unbound antigen was removed "washed" away with a buffer. The serum sample was then added to the wells. Once the antigen antibody binding was complete the un-bound antibody was washed off. Anti Ig

was then added to the wells. Once that step was completed the unbound anti Ig antibody enzyme complex was removed by washing the sample. Finally, a chromogenic substrate was added and incubated to convert the enzyme to a colored product. The colored product was then analyzed in a spectrophotometer to determine the presence of IgG1,IgG2, and interleukin 12.

ELISPOT

An ELISPOT assay for cytokine production will also be utilized for the detection of IFN- gamma. The assay was performed in wells coated with anti-IFN-gamma. The plates were incubated overnight. T cells suspended in AIM-V medium were then added. Next, the plates were vigorously washed six times and were again incubated for 2 h. The substrate Tramethylbenzidine (TBM) was added and then incubated for approximately 30 minutes. The plates were prepared for blue spot counting (the results of which will be determined through image analysis). Based on The number of blue spots per well was determined with a computer-assisted image analysis system. The frequency of positive IFN gamma producing cells per the total number of plated cells was calculated after the number of spots in control wells had been subtracted from that in experimental wells.

Flow Cytometry

Flow Cytometry is used for the purpose of quantifying the numbers of a cell having a specific marker on their membrane. A specially labeled antibody is used to bind to specific molecules and is detected through the emission of UV light. The “tag” is usually a Flourochrome antibody which can be either green or red. The tag then emits a wavelength that is visible and be recorded and plotted by means of a Flow Cytometer. Blood from the vaccinated patient will be used as the sample to be analyzed for T cells. All the erythrocytes were then meticulously removed by centrifugation. A fluorochrome-labeled antibody, specific for CD4 structure, was the added to the sample. The suspension of cells and specific antibodies was then incubated and the cells were then run through the flow cytometer. As cells pass the detector the amount of fluorescence is quantified. A fluorescence-activated cell sorter uses the fluorescence signals to separate the cells into groups based on what fluorescence-labeled antibody cells bound and if they bound at all. Since one cell passes the detector and its amount of fluorescence is quantified it can be analyzed how many cells had the CD4 molecule.

Cytoxicity

The cytoxicicty test will be conducted to determine the level of CD8 T cells (Maris 2003). The test will begin with incubating the infected cells with a 51CR tag. 51 CR is a marker that is immediately taken up by the CD8 Tcell through the cell membrane. The excess 51CR tag will then be washed. The cells will then be Incubated with 51 Cr for a period of 8 hours. The cells will then be lysed; as the cells die, 51 Cr is released. The

amount of 51 CR released from the cells can then be counted after the sample is centrifuged and dried overnight in a laminar flow hood. The dried samples can then be counted using a Microbeta liquid scintillation counter (Maris 2003).

Peripheral Blood Leukocyte Level

The peripheral blood leukocyte level (PBL) of vaccinated patients will be measured in this study. It has been documented by John N. Galgiani, MD in Tucson Arizona, that the peripheral blood leukocyte level in vaccinated patients has been abnormally low; we expect the same to be true in our pre-vaccinated patients as well. We will use this test as a safeguard to ensure that our vaccine/ adjuvant combination will not cause the patients PBL to lower and as a test that our vaccine is eliciting an immune response. First, blood will be taken from vaccinated patients. The blood samples were then allowed to sit at room temperature for 8 hours before being transferred to centrifuge tubes (Chernoff 1997). The samples were then put into a centrifuge. The centrifugal force will then separate the erythrocytes from the leukocytes. The leukocytes will then be removed and quantified using a wet smear to obtain the PBL (Chernoff 1997).

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