

Cellular Immunity against *Cryptosporidium parvum* using Subunit Vaccine

Literature Review

Cryptosporidium parvum is a parasite from the phylum Apicomplexa that is the leading cause of cryptosporidiosis in humans (Dubey 2). The parasite can infect anyone and is particularly life threatening to those with AIDS; a fact which gained it attention in the 1980's as more outbreaks among those with AIDS were seen. *Cryptosporidium* is hard to contain due to its refractory nature, which makes it indiscernible to water treatment plant filters and invulnerable to chlorine. This disease spreads through water contamination and contact with infected fecal matter. There are available treatments but no cure. For those without access to healthcare, who are undernourished, or are infected with AIDS an infection can be life threatening. This is, also, true for babies and sometimes small children since cryptosporidiosis results in excessive water loss. In the case of citizens from a small Milwaukee town *C. parvum* invaded the water supply and spread quickly to the residents. The rapidity with which this pathogen can spread, its invulnerability to normal treatment and preventative measures, and the threat it poses to those with AIDS, especially for countries with high rates of HIV infection, are the reasons that our research group has focused its attention on developing a cure for this particular pathogen.

Pathogen Structure

Cryptosporidium was first characterized as a genus by Ernest Edward Tyzzer in 1907 after noticing the protozoa in laboratory mice. Tyzzer named the strain in mice *Cryptosporidium muris* (Dubey 2-12). *Cryptosporidium* has been found on every

continent with the exception of Antarctica. The species under scrutiny in this work is that of *C. parvum* genotype I now known as *C. hominis* due to its sole infection of humans. It is important to note that another form of *C. parvum* exists, known as genotype II, the form found in bovines. *C. parvum* genotype I is a single celled protozoa that is an obligate intracellular parasite. The infective state of the organism is the oocyst that is 3µm in diameter. The sporocysts are resistant to most chemical disinfectants, but are susceptible to drying and the ultraviolet portion of sunlight. *Cryptosporidium* was first thought only to be an animal pathogen. The first human case was seen in a 3-year-old girl from Tennessee in 1976. The symptoms included severe gastroenteritis for two weeks. The species *Cryptosporidium parvum* is the infectious strain for humans. Since 1976 more and more research has linked high rates of cryptosporidiosis with HIV patients who have progressed to the AIDS phase.(Dubey)

Cryptosporidium parvum ingested as an oocyst, undergoes excystation when the suture dissolves and the sporozoite parasitizes the host. It is thought that because *cryptosporidium* can excyst in warm aqueous solutions instead of requiring reducing conditions and bile salt contact, it can also infect other areas aside from the gastrointestinal tract (Fayer 4). The anterior end of the sporozoite adheres to the epithelial surface causing microvilli to surround it and take it in through Peyer's Patches (Fayer; Armson). *C. parvum* has two schizont types which develop six or eight nuclei that become part of a merozoite, the form which leaves the host to continue infection in other hosts (Fayer 5).

Population at Risk

According to the Center for Disease Control fact sheet the first symptoms of the disease can appear within 2 to 10 days of ingestion and include watery diarrhea, dehydration, weight loss, stomach cramps or pain, fever, nausea and vomiting. While many experience these symptoms there are those who have no symptoms at all. The small intestine is the site most commonly affected, but *Cryptosporidium* infections could possibly affect other areas of the digestive or the respiratory tract (Armson). The populations most at risk are children, elderly, and HIV patients. For example, in Tanzania 17.3 percent of those infected with HIV contract *cryptosporidium parvum*. Several studies have shown that the sharing of toys and bathroom areas in daycare centers has contributed to a higher infection rate of children in this environment than any other. Those who live in areas without strict regulation on waste disposable in fields and farmlands are at highest risk during rainy seasons. Elderly patients in hospice facilities and those who care for them are at higher risk. While these individuals are listed as highest risk it is important to note that anyone who comes in contact with contaminated water or fecal matter can contract *C. parvum*.

Epidemiology

It has been documented that *Cryptosporidium* is spread primarily through fecal-oral contact, although as stated previously, it can be spread through contaminated water run off from farmland where infected cattle and other livestock are raised. Drinking and swimming in the infected water can cause outbreaks, as in the case of Milwaukee,

Wisconsin when an infected water supply caused an outbreak throughout the entire town. There is speculative evidence of airborne transmission in cases of animal care specialists inhaling near infected animals. Nevertheless, due to the close proximity of the individuals to the infected animals' fecal oral transmission has not been ruled out. Fomites and arthropods are by many considered possible forms of transmission. Furthermore, it is believed that a large number of human cases are linked to person to person contact via linens, clothing, potty chairs for children, etc.

Infection can occur in areas of the esophagus the gastrointestinal tract and the rectum, usually starting in the lower small intestine. Other areas include: the gall bladder; bile ducts; pancreas; and respiratory tract have been known sites of infection in previous cases. An infection that induces symptoms of diarrhea can result in as many as 71 stools and 12-17 liters a day. This is not always the case, but despite this it has been reported that for those infected the infection significantly disrupts daily routine and working conditions are not always conducive to such a disease. Other symptoms can include abdominal cramps, vomiting, low grade fever, general malaise, weakness, fatigue, loss of appetite, nausea, chills and sweats. These symptoms can last anywhere from 2 to 26 days. Cryptosporidium affects individuals in a variety of different ways that marked on a scale would associate the most afflicted cases with chronic life threatening illness and at the other would demonstrate asymptomatic individuals (Armson).

Disease Mechanism

In the case of the most common infection site, the area of the small intestine, cryptosporidium will use as its entrance site the mucosa of the small intestine. This area

covered by a host of villi for absorption of nutrients is not just an ideal site for cryptosporidium to invade the immune system; it is the prime entrance site for a variety of opportunistic pathogens. The information on the mechanism of disease of cryptosporidium has come primarily from research on mice, specifically mice with severe combined immune deficiency (SCID), a condition which renders the mouse even more immunodeficient than nude mice. What is known so far is that *Cryptosporidium* uses gp900 and pg40/15 complex, attached to the sporozoite surface, to attach and invade the epithelium of the mucosa.

Treatments or Vaccines

For a long time only the symptoms of diarrhea were treated and no antibiotic existed for Cryptosporidiosis. However, a new drug called Alinia, nitazoxanide, has recently been approved by the FDA as an antiprotozoal treatment. The drug is an antibiotic taken orally every 12 hours for those experiencing diarrhea due to a protozoa infection. Some common side effects, listed by The Mayo Clinic, include stomach pain, diarrhea, headache and vomiting and in rare cases a person may experience appetite increase, bloating, discolored urine, yellow eye discoloration, dizziness, enlarged salivary glands, excess gas, fever, itchy skin, loss of appetite, nausea, sweating, fatigue weight loss, or general discomfort.

VACCINE DESCRIPTION

Cryptosporidium parvum is an intestinal parasite that is commonly transmitted via infected water. *C. parvum* typically causes diarrhea but can become a very severe

problem in immunocompromised patients. Because of this potential risk, a subunit vaccine will be developed against *C. Parvum*. A subunit vaccine is developed from purified antigens of the pathogen. Genes that contain the appropriate antigen are isolated from the genome and then inserted into a host cell where transcription and translation occur in order to produce large quantities of the purified antigens. The benefit of the subunit vaccine is that there is no risk of the patient becoming infected with the organism because only pieces of the organism are being utilized rather than the whole organism, as is the case with attenuated vaccines. This is important because the vaccine will be safe to administer to immunocompromised patients who run the largest risk of severe disease if infected with *C. parvum*. The disadvantage of the subunit vaccine is that it does not effectively elicit an immune response on its own. An adjuvant will be used to help direct the vaccine and achieve the necessary immune response for the vaccine to be effective. The adjuvant that will be attached to the vaccine is an immunopotentiating reconstituted influenza virosome (IRIV). This adjuvant was chosen because it is extremely safe and very effective at eliciting immune responses. It is able to do this because the virosome retains some of the infectious glycoproteins, most notably hemagglutinin. Hemagglutinin induces membrane fusion and when the virosome fuses with the endosome of the host cell the antigens on the surface will be degraded and presented in the form of Class II MHC (Glück et al., 2005).

C. parvum begins its life cycle in the intestinal lumen after the infective oocysts are ingested. The oocysts release sporozoites that are free in the lumen for a short time and then they infect the epithelial cells where the remainder of the life cycle takes place. The sporozoite stage was selected to be the target for the vaccine because it is free in the

intestinal lumen and is not in the host cell making it more susceptible to phagocytosis and digestion by Peyer's patch M-cells. This makes it more likely that the components of the pathogen will be presented to T-cells by a MHC class II restricted path. The effect of the CD4+ T-cells that respond to the MHC class II are very important in the termination of the infection (Riggs, 1999). There are some problems associated with targeting the vaccine at the sporozoite stage, the first being that this stage occurs very quickly, the vaccine needs to be able to work quickly to neutralize the pathogen before infection of the epithelial cells occurs. An oral vaccine will be used to combat this problem because it is the quickest route to the epithelial cells in the lumen and the vaccine should have the amount of time necessary to illicit the needed immune response before infection of the host cells occur. Another problem is that effective amounts of the neutralizing antibodies need to be present at all areas of infection because if even one epithelial cell is infected the life cycle can complete and clinical disease can occur.

An important aspect of an effective vaccine is selecting antigens that will achieve the immune response needed to fight the infection. Because the sporozoite stage was selected as the target for the vaccine, it is important to select antigens that will neutralize the sporozoite and inhibit invasion of the host cells. Previous research has indicated that CP15 and P23 are the most promising antigens for the development of a vaccine against *C. parvum*. CP15 has been previously investigated as an antigen for cryptosporidiosis and has been found to be effective at inducing both a local and a systemic humoral immune response. P23 is a surface pellicle glycoprotein that plays a significant role in the pathogenesis of the infection. P23 also have neutralization-sensitive epitopes that

reduce the infection and therefore make it an important antigen. It is also proficient at inducing a cell-mediated immune response (Boulter-Bitzer et al., 2006). Another important aspect of both of these surface proteins is that they have unique amino acid sequences that are help to distinguish between these antigens and the host, therefore reducing the risk of not having any response because the antigens weren't recognized and also reducing the risk of the immune system turning on the host cells.

Description of Immunity Assessment

Several tests can be performed to demonstrate antigen specificity and other qualities of adaptive immunity. The four parameters to be met after vaccinations are: immunological recognition, self/non-self discrimination, immunological specificity, and immunological memory. The use of specified assays utilizing serum T-cells, should be able to test each of these four requirements for conferred immunity.

The purpose of the oral vaccine is to allow for mucosal immune responses to occur, rather than systemic immune responses that can be seen in injection vaccines. Eliciting mucosal immunity is key in fighting off cryptosporidium infections as crypto infects the intestinal cells of the GI tract. (Riggs, 2002) Cellular and humoral immunity will be induced, and tests will be run to quantify cytokine, cellular, and antibody levels post vaccination.

Upon successful completion of animal testing oral vaccines will be given to people in endemic regions of cryptosporidium outbreaks, or people who are at high risk of contracting the disease, such as veterinarians. A blind study will be done, with roughly

50% of the participants' receiving the vaccine and the other 50% receiving a placebo. This will be done to quantify differences between the negative controls and the vaccinated participants immune response. Mucosal and Serum levels will be checked the day of vaccination prior to vaccination for antibodies against *Cryptosporidium*, and everyday for fifteen days. Fifteen days was chosen for the trial run, as it should give the immune system ample time to take in antigen and form a proper cellular and humoral response with active memory cells that can be quantified. Mucosal swabs of the nose and mouth will be taken to determine levels of antibody present. Antibodies that will be screened for will be IgA, IgG, and IgM. IgG will be a necessary antibody as well although IgA will be the dominant antibody we are testing for. (Chappell, 1999) Serum and mucosal swabs will be checked to quantify antibody levels. Proper antibody levels will be needed to help cellular immunity in resolving an infection with *Cryptosporidium parvum*. (Angeles, 2002) Antibodies will assist in targeting extracellular sporozoites and merozoites as they leave one cell to infect another. These antibodies will be able to neutralize *Cryptosporidium* by binding to membrane receptors that bind and aid in cell invasion.

The first response that will be monitored for will be the innate immunity. M-cells and dendritic cells located in the gut will take up and begin presenting our foreign antigen, cryptosporidium receptors CP15 and CP15/60. Macrophages will be important, as they will begin secreting cytokines IL-1, IL-6, IL-8, IL-12, and TNF α . These cytokines will promote increase vascular permeability at the site of infection to allow for more leukocytes to enter into the tissues. Endothelial cells lining the neighboring blood vessels begin expressing adhesion molecules, which allow for chemotaxis to occur.

TNF α also increases cellular expression of adhesion molecules, which facilitate diapedesis. (Mullapudi, 2007)

Adaptive immunity will take over a few days post-vaccination. Cytotoxic T cells will be the main force in eliminating the infection by recognizing infected cells and triggering cell death. Plasma cells will secrete the needed anti-crypto antibodies IgA, IgG, and IgM. T-helper cells play an important role in assisting in infection resolution by secreting cytokines INF γ . INF γ is essential in that it triggers macrophages to present more antigen on their MHC and eliminate the pathogen more efficiently (Janeway, 2001) This will allow for faster resolution of infection.

In order to determine if a vaccine trial was successful our vaccine recipients must illicit a strong enough humoral and cellular immune response as well as generate memory cells. Specific test will be used to measure vaccine efficacy. Enzyme-Linked Immunospot (ELISA) will be used to measure the ability of memory T-cells (CD4 and/or CD8) to make cytokines based on antigen specificity. After T-cell exposure to antigen, antibodies (specifically IgG and IgA) will be added and after binding specific cytokine, stained cytokine-producing cells can be counted. The ELISA test will be performed 5 days after initial challenge and a successful trial will be one with a high amount of particular antigen-antibody complexes (It should take roughly 5 days for an adaptive immune response). A potential problem with ELISA is the possibility of obtaining false positives. False positives can result from cross-reactions with other present molecules, impure reagents, etc. Recurrent trials with sterilized test kits should overcome this problem and deliver accurate results. (Janeway, 2001)

Further tests to study adaptive immunity will be a lymphoproliferative response test (LPR), intracellular cytokine staining (ICS), and flow cytometry. LPR tests are difficult to analyze but can be used to test the ability of memory T-cells to proliferate upon antigen exposure. Lymphocytes are treated with antigen for roughly 2-4 days, which is sufficient time for cell proliferation. Radioactive thymidine is then added to the culture medium for 4-6 hours. The incorporation of the labeled thymidine is indicative of cell proliferation. LPR tests will test for immunological specificity but results take roughly 5-6 days so the test will be supplemented with other tests. (Decker, 2007) ICS, which is similar to ELISA tests in theory, utilizes a substance that traps synthesized cytokines within T-cells. T-cells are treated with an inhibitor of protein export, therefore, T-cells can produce cytokines but cannot secrete them. Intracellular cytokine concentrations are allowed to reach a high concentration and then cells are treated with a mild detergent (Saponin, for example). The detergent permeabilizes the cell membrane and Fluorochrome labeled antibodies can interact and form complexes with the cytokines. ICS can be used in conjunction with flow cytometry to quantify the amount of activated T-cells as well as to measure the amount of antigen-specific antibodies. The fluorochrome labeled antibody-antigen complexes in the suspension-vibrating will separate into individual droplets and the laser beam detector can quantify the amount of fluorescence in each intensity level. (Vogel, 2002)

Flow cytometry will be used to quantify cytotoxic T cells specific to our antigens, glycoproteins P23 and CP15. T cells will be separated out of patient serum and bound with a MHC1 tetramer with fluorochrome. The anti-crypto MHC1 tetramer with fluorochrome will bind to the membrane receptors on the cytotoxic T cells that are

specific against *Cryptosporidium*. They will then run through a flow cytometer to quantify cytotoxic T cells that are anti-crypto. The purpose of the MHC 1 tetramer with fluorochrome is to allow for better binding with host cells than an antibody would, so that we may better quantify them. (Janeway, 2001) We expect a higher mean fluorescence for a successful trial; which indicates a higher presence of CD8+ anti-crypto cells post vaccination. (Angeles, 2002) Positive test controls will be T cells with known positive *Cryptosporidium* MHC receptors. Negative test controls will be another cell type expressing MHC2, which will be unable to bind to our anti-human MHC1 tetramer.

The purpose of an oral vaccine is to allow for proper mucosal immune responses to occur. A potential problem with an oral vaccine however is how to determine if the subject received enough of the vaccine by absorption in the lumen of the small intestines, rather than the vaccine being passed out of the system, or being destroyed by the high acidity of the stomach. (Riggs, 2002) In order to correct this potential problem pilling of the vaccine could potentially reduce the destruction of the antigens and adjuvant by the low pH in the stomach. Another potential problem is that many one dose vaccines are not strong enough to elicit an immune response. By using virosomes we hope to avoid this problem all together.

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