

Chagas Disease DNA Vaccine for the Americas

Chagas disease affects thousands of people each year. With many people immigrating to the United States every year from areas of Latin and Southern America where Chagas disease is prevalent, the spread of a serious public health issue could develop in the United States within several years. Cases of Chagas disease are already appearing in the southern United States. One of the main problems is that infected people are coming into this country without being screened for disease. The threat of spreading infection occurs when these people donate blood or organs. Screening for Chagas disease has not been a requirement thus far; however, since several cases of the infection in the United States have been reported after receiving infected blood or organs, testing has become more widespread.

Epidemiology

Chagas disease is caused by the flagellated parasite *Trypanosoma cruzi*. The parasite is transmitted by hematophagous assassin bugs, also known as triatomids. The parasite can live in a macrophage without being killed. *T. cruzi* escapes the immune response by hiding out in the heart tissue.

Chagas disease currently affects sixteen to eighteen million people in Latin America, with 25% of the population at risk for acquiring the disease. Every year Chagas disease kills around fifty thousand people. Cases of the infection have reported in southern US to southern Argentina, mostly in the poor rural areas of central and southern America. In the poor rural areas the bug that transmits the disease gets into the buildings constructed of mud, adobe, and straw. The bug sleeps and hides in the walls and roof during the day, and then at night they emerge to feed on the building's inhabitants.

Transmission of the *Trypanosoma cruzi* to mammals can occur by various methods. *T. cruzi* is carried in the gut of the bloodsucking triatomid. The bug first bites an infected animal or person, then goes on to bite another mammal. Transmission from bug to mammal happens when the bug defecates on the mammal's skin after taking up a blood meal. Because the parasite is carried in the feces of the bug, it is able to enter the mammal through either the bug's bite site or the mucous membranes. People can also get infected from receiving a blood transmission or organ transplantation from an infected person. The parasite can also cross the placenta; therefore a mother can give it to her unborn child. The disease may also be acquired by consuming uncooked meat that is infected with the parasite.

Symptoms of this disease include a red and swollen area around the bite site, swelling of an eye, fever, irregular or rapid heartbeat, and enlarged lymph nodes. Also, in the chronic stages of this disease a patient may exhibit signs of heart failure or cardiomyopathy, which can result in death.

***Trypanosoma cruzi* Life Cycle**

The life cycle of *Trypanosoma cruzi* takes place in both the bug vector and infected mammal. The triatomine insect becomes infected by feeding on blood that contains the parasite. The trypomastigotes are ingested and move to the midgut of the triatomine. Trypomastigotes change into dividing forms, epimastigotes, in the insect gut lumen. Epimastigotes typically pass from the midgut to the hindgut but some may remain in the midgut. They reproduce asexually by binary fission in the midgut or hindgut and migrate further along the hindgut and rectum where they attach to the cell wall using their flagella. The epimastigotes transform into metacyclic (infective) trypomastigotes while

attached to the hindgut or rectal cell wall. Trypomastigotes remain in the rectal lumen until the triatomine insect takes its next blood meal. While taking a blood meal, the insect deposits infected feces onto the skin of a new host. The trypomastigotes infect the host through the bite wound or by crossing mucous membranes. The trypomastigotes penetrate various non-phagocytic and phagocytic cells, typically around the wound site. Once into the host cell cytoplasm, the trypomastigote loses its flagellum and transforms into an amastigote. The amastigote divides by binary fission within the host cell cytoplasm and multiplies to form a pseudocyst. After about 5 days the amastigotes transform into small C-shaped trypomastigotes, and eventually, the pseudocyst ruptures, releasing the trypomastigotes. Some trypomastigotes circulate in the blood and infect host cells. Other trypomastigotes circulating in the blood may be taken up by a feeding triatomine vector to continue the cycle.

Available Treatment

The treatment available for Chagas disease does not work very well. There are some drugs available for the acute stage of infection; however, there have been reports of resistance to these drugs. There is an antifungal medication that has been used as a second-line drug, but it is costly and is highly toxic. In the chronic stage, there is no way to cure the patient. Some may get pacemakers due to heart failure and arrhythmias. Heart transplant is another option, but there is a possibility that the parasite would then infect the new heart due to the immunosuppression that the patient goes through after receiving a transplant. Researchers are currently working on developing a DNA vaccine.

We chose to develop a vaccine for Chagas disease because it affects millions each year. There is no good way to treat this disease, and even if there was, the people that are affected the most can't afford the appropriate health care. If a vaccine was developed, it would not only help the people of Latin America, it would also prevent the spread of infection in the United States due to the increasing threat of Chagas disease.

Immune Response

T. cruzi enters the body during the trypomastigote phase of its life cycle. This trypomastigote stage is also the stage in which *T. cruzi* is exposed to the immune system in the blood stream. The other phases of the life cycle are spent either in the vector insect or inside cells within the infected person. This means that the immune system is exposed to the trypomastigote phase more than any other and it is this form that the parasite is first seen by the immune system.

Once the trypomastigote enters the body it encounters macrophages and non-phagocytotic cells. The parasite is able to enter cells of both types and reproduce within their cytoplasm without being phagocytosed. This is one of the ways in which *T. cruzi* is able to evade immune defenses, but it also poses problems for antigen presentation. If macrophages are not able to phagocytose the parasite, it will not be able to present antigen as readily. Without this presentation, a specific immune response is harder to make. However, *T. cruzi* specific T cells have been found in infected person, which is only possible if the parasite antigen is being expressed on class I MHC (Marin-Neto, 2007). This means that at least some of the trypomastigote parasite antigens are being presented to T cells by dendritic cells within the thymus.

Other evidence suggests that parasite antigens may be expressed on class I MHC. When *T. cruzi* transforms from trypomastigote to amastigote within the host cell cytoplasm it reduces its size by up to 90% (Michailowsky, 2003). Many proteins are shed

by the parasite in this process, which are then left in the cytoplasm of the cell. The cell proteasome then degrades these proteins into peptides, which are transported to the E.R. then the golgi and finally out to the outer cell membrane on class I MHC. This explains how the immune system can elicit *T. cruzi* specific CD8 cells even though the parasite can avoid phagocytosis by macrophages.

Further evidence for CD8 activation comes from a study that showed *T. cruzi* was a major inducer of IFN- γ (Abel, 2001). IFN- γ , when used in conjunction with CD40 ligand, helps to activate macrophages. This study also presented evidence that inflammatory cytokines released during a *T. cruzi* infection may play a role in cardiomyopathy that accompanies chronic Chagas disease. However, since the *T. cruzi* parasite is able to evade being phagocytosed by the macrophages and is able to use the macrophage as a place to reproduce, the activation of macrophages may not be that beneficial in the control of the *T. cruzi* infection, as it will only provide the parasite more hosts in which it can reproduce.

Although Th cells and other regulatory immune cells are also activated in response to a *T. cruzi* infection these have been shown to have a limited role in the control of the infection (Kotner, 2007). It was thought that these regulatory cells might inhibit a hyperactive immune response, which kept the parasite from being completely eliminated from the body. This was shown not to be the case as these helper cells have a limited role in the infection. This supports the idea that Th cells, which help activate macrophages, might actually be helping the *T. cruzi* parasite by sending in more macrophages with which the parasite can infect and reproduce in.

It is suggested that immunodominant regions in surface molecules such as TSA-1 may provide a mechanism for the parasite to evade the host immune response by directing the response away from epitopes that have the potential to elicit a reaction that is damaging to the parasite (Wrightsmann, 1994). The TSA-1 (trypomastigote surface Ag-1) is a major surface antigen of the bloodstream trypomastigote stage of *Trypanosoma cruzi*.

The most serious consequence of a *T. cruzi* infection is heart disease and there is considerable debate about the mechanism involved in the pathology (Dumonteil, 2004). Some evidence points towards damage to heart tissue associated with replication of intercellular amastigotes (Tarleton, 1999) while other studies show that autoimmunity caused by parasite antigens mimicking host proteins is responsible for tissue damage (Leon 2001). The prevailing thought was that Chagas disease has autoimmune etiology because signs of disease are present in tissues where the parasite is not located. However, more sensitive techniques including PCR analysis of infected and damaged tissues have shown that there is a uniform correlation between tissue damage and presence of parasites. This does not rule out the possibility that autoimmunity plays a role in the damage of host tissues, but it appears that this autoimmune response is only found in the presence of the parasite.

DNA Vaccines

A DNA vaccine is comprised of a DNA plasmid carrying an antigen-coding gene or epitope. The plasmid has the ability to transfect cells in vivo, resulting in the presentation of an antigenic protein (Whalen, 1996). The body can then mount an immune response against the antigen, creating memory cells and antibodies specific for that antigen. Like any other vaccine, re-exposure of the host to the pathogen will immediately induce an immune response and allow for rapid removal of the pathogen. Along with the plasmid,

two other elements are required for a successful vaccine. The first is a DNA promoter sequence to enable transcription of the protein gene once it enters cells. The other is a cytokine adjuvant, which aids in a more effective immune response. Currently cytokines such as IL-12 or IL-23 show promise as useful adjuvants in DNA vaccines (Williman et al., 2006). Once all three elements are combined, the vaccine can be injected directly into muscle cells (Egan et al., 2006).

Benefits of DNA Vaccines

The development of DNA vaccines for immunization comes with several benefits. One benefit is that DNA vaccines are more easily manufactured than other types. One reason for this is that most plasmids can be made the same way, so plasmids carrying different epitopes can all be made using the same technique (Whalen, 1996). Also, because DNA is a stable structure these vaccines are less resistant to temperature, making them easy to store and transport (Whalen, 1996). In general, vaccines tend to be the most cost effective way to control the spread of infections and with the easy manufacturing of DNA vaccines, costs for treatment using this method are very low (Boyer et al., 2006).

DNA vaccines can induce both a cell mediated and an antibody response (Egan et al., 2006). Because this form of treatment activates both responses, it can be used for intracellular and extracellular pathogens. For a cell-mediated response, the plasmid is first taken up by a dendritic cell. After transcription and translation, the newly formed protein is degraded into peptide fragments that can then be presented to cytotoxic T cells by MHC class I. The T cells will then seek out cells infected with the specific epitope and lyse the cell before infection can spread.

For an antibody response to occur the plasmid must first be taken up by a cell other than a professional antigen presenting cell. The protein is made and released into the extracellular environment. Now the protein can be taken up by an antigen-presenting cell, degraded, and presented to a helper T cell by MHC class II. The helper T cell will activate proliferation of B cells specific for the antigen. The B cells will produce antibodies against the pathogen and memory cells to protect the individual against future infections.

In the case of *T. cruzi*, the ability of a DNA vaccine to induce a cell-mediated response is essential to eradicating an infection. Because *T. cruzi* is an intracellular pathogen, antibodies are of little use in eliminating the parasite. An additional benefit is the fact that DNA vaccines allow the inoculation of live particles, which are better for activation of CD8 cells (Boyer et al., 2005). The science of DNA vaccines has also shown success with both small and large sized mammals. Initial trials of various DNA vaccines have given positive results in mouse models and, more recently, successful vaccines have been developed for larger mammals such as horses (Weiner, 2006). In time DNA vaccines may soon be ready for human use and *T. cruzi* infections would be an ideal use for such treatment.

Route and Timing of Vaccine Administration

In order to prevent possible infection of the *T. cruzi* parasite, the time of administration of the DNA vaccine would most likely be in a human's youth before any infection. The route of choice for this vaccine would include muscle injection, similar to most DNA vaccine treatments.

Immunological Tests

Possible epitopes that can be introduced via DNA vaccine include Ssp-1, Ssp-2, and Ssp-3 of the *T. cruzi* cell surface. The Ssp-1 epitope was observed on glycoproteins that are approximately 100, 120, and 150 kDa and the Ssp-2 epitope was identified as a 70 kDa surface membrane protein. These three epitopes are applicable because it has been observed that trypomastigotes that had recently emerged from mammalian cells expressed Ssp-1 and Ssp-2. However, during extracellular incubation these epitopes gradually decreased. It was also observed that Ssp-3 increased during the first six hours of extracellular incubation and then gradually decreased, but was still detectable after forty-eight hours (Burleigh and Andrews, 1995).

To demonstrate the efficacy of this vaccine, immunological tests including the ELISA would be applied. The ELISA test would be utilized to show that the epitopes, i.e. Ssp-1, Ssp-2, and Ssp-3, introduced to the body via DNA vaccine have been presented by the human's MHC class I. In order to do so, an antibody made specifically for these three antigens would be developed in any animal of choice. After evenly coating the microtiter vessel with the primary antibody linked to the *T. cruzi* epitopes, human cell epitopes, which should be expressing the epitopes of the *T. cruzi* that were introduced in the DNA vaccine, are combined in the same vessel. Once the antigen has bound and the unbound antigen has been washed away, enzyme-labeled antibody that is specific for the *T. cruzi* epitopes is added, which should make a sandwich in the order of antibody, antigen, and enzyme-labeled antibody. Using a chromogenic substrate for the enzyme used to label the antibody, the color of the assay can be used to determine the presence of the antigen within the vaccinated human's body. This test for specific epitopes within the vaccinated human should be applied a few days to two weeks after the DNA vaccine has been administered in order to allow the body to familiarize itself with the epitopes and express them on its own cells.

Another possible test to verify the effectiveness of the DNA vaccine is an ELISA to detect the levels of TNF alpha present in the subject's body. The role of TNF alpha in the body includes the activation of natural killer cell cytotoxicity, enhancing generation of cytotoxic T-lymphocytes, and the activation of natural killer cells to produce interferon gamma. Although, TNF alpha is produced by a variety of cell types in the body, activated macrophages represent the dominant source. Therefore, the levels of TNF alpha during infection should be higher in vaccinated subjects than in non-vaccinated subjects because this parasite destroys macrophages, which would express the TNF alpha cytokine. In order to identify the difference in TNF alpha levels, the results of an ELISA for a subject not infected with the *T. cruzi* parasite and an infected subject would need to be compared. To test for TNF alpha levels, the sandwich ELISA would be applied. With this method, an antibody developed specifically for TNF alpha in any species would be bound to the microtiter plate surface. A blood serum sample of the subject would then be incubated in the same vessel, and washed off for any excess, unbound antigen. Following this step, the primary antibody, specific for TNF alpha, with an enzyme label would be added to the well in order to bind and form a sandwich of the antibodies and antigen in the presence of TNF alpha. After the addition of a chromogenic substrate to activate the color in the assay, the presence and degree of concentration of TNF alpha from the subject can be identified. If the antibodies are complementary to the TNF alpha, a darker color of the assay will indicate higher levels in TNF alpha, which should be the

result for the non-infected and vaccinated subject, and a lighter color in the assay should be relative to the infected subject's results.

Because this infection involves an intracellular parasite that is out of reach of antibodies a cell-mediated response is required which includes cytotoxic T lymphocytes and helper T cells. However, the antibody that would most likely be synthesized by default of the body's immune response would be IgG due to its common functionality throughout the body. In order to identify the presence of the IgG *T. cruzi* epitope specific antibody, an ELISA assay is required. Using the indirect ELISA method to detect specific antibody, a microtiter plate would initially be coated with a purified mixture of Ssp-1, Ssp-2, and Ssp-3. After washing away any unbound antigen and washing the plate with a solution of generic powdered milk to prevent nonspecific binding of the antibody, the serum sample from the tested subject is added. After incubation, an enzyme-labeled secondary antibody specific for the IgG antibody, most likely specific for the constant regions of the IgG antibody, from any animal would be added to the microtiter vessel. Lastly, a chromogenic substrate for the enzyme linked to the secondary antibody would be added in order to show color in the presence of the specific antibody that is being assayed. With a positive result, the assay will show color and determine that the IgG antibodies specific for the Ssp-1, Ssp-2, and Ssp-3 are present within the vaccinated subject. A problem with this assay may include the specific isotype of antibody being assayed. If the antibody specific for the antigen is a different isotype than IgG, then the assay will show no color and no result, which would be a false negative for the presence of *T. cruzi* epitope specific antibodies in the subject's body.

As a means of testing B cell memory, the antibody levels should be confirmed at various spans of time after the vaccine has been administered to the subject. By doing so, the production of *T. cruzi* epitope specific antibodies can be quantified indicating the length of the B cell memory for this vaccine. This test would again be achieved using the indirect ELISA method that is used to detect the presence of a specific antibody using the *T. cruzi* epitopes of Ssp-1, Ssp-2, and Ssp-3.

Potential Complications

As previously mentioned, the epitopes present on the *T. cruzi* parasite are not constant. Throughout its lifecycle within the mammalian body, the epitopes on its surface are constantly changing, appearing, and disappearing. Another problem with the *T. cruzi* parasite includes its heterogeneity among forty-three different clones identified (Michel Tibayrenc, et al, 1986). This complex multiclonal structure indicates the ability of the parasite to readily adapt, and therefore, with a common vaccine it may evolve to become resistant. The solution to this problem could be to investigate the more common epitopes of the *T. cruzi* and observe their presence at various stages of the parasite. With this information, common epitopes between the different clones observed can be identified and used in the DNA vaccine depending on their inconstant presence on the parasite. Another solution could be to develop multiple antibodies against various epitopes present on the parasite in order to protect the mammal at all stages of the *T. cruzi* lifecycle and in case of resistance.

Criteria for a Successful Trial

In order to determine whether this vaccine is successful in preventing the infiltration of the *T. cruzi* in the body, positive results must be seen with the expression of *T. cruzi* epitopes within the vaccinated cells as well as inhibition of binding of the *T. cruzi* to the

macrophages within the body due to the presentation of these epitopes. By way of ELISA methods, positive results of *T. cruzi* epitopes in human cells and high levels of TNF alpha in infected, vaccinated subjects would suggest success with this vaccine.

Length of Study

The length of time required for this study would be approximately within the year ranges. The estimation of this time span is based on the amount of time required to sequence and express specific proteins, to obtain an immune response from the subjects, to perform the previously mentioned tests, as well as to repeat the process in order to determine the error of the vaccine.

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