

Lipid Compartmentalization of GP90 Antigen To Induce Immunity to Chagas Disease

Introduction

American trypanosomiasis is an infectious disease caused by the protozoan parasite *Trypanosoma cruzi* (Levy et al, 2006). The disease was first identified by its dividing epimastigote and the various metacyclic trypomastigote forms. Dr. Carlos Chagas was the Brazilian doctor who first identified it in 1909 (Teixeira, 2006). The disease is transmitted to humans and animals through an invertebrate called the “kissing bug,” a triatomid of the family Reduviidae. The insect is also known as an assassin bug due to its tendency to pierce the blood-filled abdomen of other members of its species. The insect vector bites an infected animal or person, and then transmits the parasite once it travels to another host for a blood meal. *T. Cruzi* is not transmitted through the blood of the vector; it is transmitted through feces deposited on the skin near the site of penetration (Chagas, 2006). The host becomes infected when the irritation causes them to scratch or rub the infective fecal matter into the open wound. The insect feeds without being detected due to its very sharp mouthparts and speeds the process by releasing proteins called nitrophorins which release nitric oxide promoting vasodilation, and preventing inflammation. The parasite is most often maintained in a sylvatic cycle between small wild animals. Blood transfusions are also a source of infection in insufficiently regulated blood banks of South America.

Mechanisms of Disease

Ecology

T. Cruzi is most commonly found in Central America, South America, Mexico and the southern areas of the United States. It extends as far north as 42 degrees and as far South as 42 degrees, which is southern Argentina and Chile (Teixeira, 2006). Within these areas, 11 million people are said to be infected with the disease, many of whom do not know it (Chagas, 2006). Of these 11 million, as many 30% will develop chronic symptoms of the disease. Migration of people from rural areas of the Americas has caused the disease to change from a rural problem to a periurban problem. The poor economic state of these countries works to promote and maintain the infection at an endemic level. The insects responsible for transmitting this disease prefer to live in thatched and adobe houses where they can hide in cracks and come out to feed during the night. The poor living conditions of many South American countries facilitate the presence of these insects. The medical impact of chronic Chagas disease has a large enough impact to require the help of a vaccine. In Brazil alone, 10% of the population develop the chronic disease and require medical treatment costing in the range of 250 million dollars. 75,000 Brazilian workers miss work resulting in great personal and economic losses.

Pathogen Structure

Trypanosoma cruzi is an intracellular protozoan parasite that infects a variety of different animal hosts. In its infectious form, the life cycle involves both insect and human hosts. Its life cycle consists of three stages: spherical, reproducing, and intracellular amastigotes in vertebrates, extracellular, reproducing, and motile epimastigotes in invertebrates, and highly motile, non-reproducing extracellular trypomastigotes in both. Each form has a flagellum, although the size of the flagellum varies by life cycle stage. The more motile stages have a flagellum as long as 20 μm , while the intracellular amastigote's flagellum is as short as 1 μm . The family to which *Trypanosoma cruzi* belongs is called Apicomplexa and is defined by the presence of the kinetoplast, a single large mitochondrion containing a large amount of DNA enhancing its transmission by enabling increased survival in the vector (Engmen, Tyler 2001).

The life cycle begins when an insect host bites an infected vertebrate and ingests trypomastigotes. These transform into epimastigotes in the lumen of the intestine and reproduce. They then transform into metacyclic trypomastigotes as they pass through the digestive tract, and exit in the feces of the insect host (Engmen, Tyler 2001). If the insect's feces are left on a vertebrate host after the insect bites, the trypomastigotes may enter the wound and proceed to the vertebrate's bloodstream. Circulating macrophages engulf the pathogen yet are unable to destroy it as a result of *T. cruzi*'s ability to escape the phagosome before lysosome fusion (Freire-de-Lima et al, 2001). The trypomastigotes then transform into amastigotes once escaping the phagosome and infect vertebrate tissue/nerve cells, with a preference for muscle cells. Colonization of organs eventually causes megasyndrome involving the enlargement and denervation of vital organs such as the heart. The amastigote forms then transform into bloodstream trypomastigotes and swim vigorously within the cell to facilitate rupture of the host cell's plasma membrane, freeing the trypomastigotes to infect other cells. The transformation from amastigotes to trypomastigotes is not uniform among all *T. cruzi* within a cell, meaning that some organisms are still in the amastigote form when the cell ruptures. Phagocytes may be infected by ingestion of amastigotes, which can break out of their vesicle once ingested. This ability also makes cytotoxic immunity ineffective against *T. cruzi*, because the amastigotes freed due to host cell death are still able to infect more phagocytes (Andrews et al, 1995).

The surface proteins on trypomastigotes play a major role in their ability to invade host cells. Unlike many other intracellular pathogens, *T. Cruzii* plays an active role in the invasion of host cells, even to the extent of using ATP-driven processes to actively invade. Several of the cell surface molecules involved in host cell binding and invasion are glycoproteins, specifically gp90 and gp82. Treatment of the parasite with kinase inhibitors and trypsin dramatically decreases their ability to infect host cells. The fact that the parasite is able to invade a wide variety of vertebrate cells indicates that the receptors on the pathogen surface may not be very specific (De Souza, 2002).

Treatment

There are two primary approaches to therapy which have the capacity to prevent a terminal infection with *T. cruzi*. An antiparasitic treatment early in the stage of infection is useful in killing the parasite although it does offer some benefit in later disease stages. The Center for Disease Control is the organization most knowledgeable about Chagas disease treatment in the United States. The other form of treatment is symptomatic treatment which is aimed towards managing the signs and symptoms of infection. This treatment is helpful for people who have chronic cardiac and intestinal problems, and often involves aids such as pacemakers and medication (Chagas, 2006).

The most common prescription drugs used against this disease are Nifurtimox (4-(5-nitro-furylidenoamine)-tetrahydro-4-4-1,4-thiazine-1-1-dioxode) and o-benznidazole (N-benzyl-2-nitroimidazolacetamide). The drug cytotoxicity is the eliminating agent of the parasite and is attributed to their chemical structures which release electrophilic radicals on enzymatic nitro-reduction. The free radicals include the nitro anion, H₂, K₂O₂ and hydrogen peroxide. The nitro group maximizes mutagenic potential by binding DNA and forming adducts. The radicals may also be harmful to some host cells so the use of nitroderivatives in the treatment of Chagas disease has limited success. *T. cruzi* is not eliminated by this drug in the majority of cases. The difficulty of this disease is the fact that the treatments available do not offer much benefit, as indicated by electrocardiogram labs which indicate very little difference between those treated for the disease and the control group given a placebo. This indicates that patients treated with these drugs are not prevented from the progression of the disease resulting in severe heart lesions. In one study it was found that mortality within treated and untreated groups were similar ten years following chemotherapy (Teixeira, 2006).

Mechanism of Disease

Chagas disease is the leading cause of heart disease in Latin America, and is caused by the parasite, *Trypanosoma cruzi* (Doyle et al, 1998). The infection is transmitted through the fecal material of the *Triatoma infestans* vector, also known as kissing bug. The parasite survives in macrophages and avoids destruction by escaping the phagosome before a lysosome can fuse. The trypanosome can live in a variety of cells, including macrophages, muscle cells within the heart, and areas in the digestive tract. The infection multiplies within the cytosol and muscle tissue and can often result in mega-colon, mega-esophagus, and cardiomyopathy (Kelly 2000). The parasite also evades the immune system with a racemase enzyme which produces proteins resistant to proteolytic cleavage by the host cells. The major cysteine protease of *T. cruzi* is cruzain, and when cruzain is inhibited growth and differentiation are prevented (Doyle et al, 1998).

Diagnostic Tests

Elisa

An Elisa test will be used to detect the production of antibody in response to the *T. cruzi* infection. The indirect ELISA will detect antibodies in patient serum. The gp90 glycoprotein coat on the surface of the parasite will be the test antigen that will bind to the antibodies present in the serum. The serum sample will be collected from mice receiving the vaccine. The first step is to secure the gp90 glycoprotein to the microtiter plate. The serum sample will be tested for specific antibody (IgG) that will be added and left to bind to the antigen. An anti-Ig from a rabbit sample is added and will bind to the Fc region (gamma chain). The Fc of the anti-Ig will have an enzyme displaying a chromogenic colored product if a reaction takes place between the enzyme and substrate. The color intensity represents the concentration of antibody present in the sample. Also, positive and negative controls must be done to validate the results. A positive control will describe the interaction of the antibody with the antigen. A negative control can be made by omitting the antigen or by adding another antibody in order to ensure that it will not also bind to the antigen and give a false positive result.

Flow Cytometry

Detection of T cell immunity including helper and cytotoxic T cells specific for *T. cruzi* antigens can be determined using flow cytometry. The production of T cells are very important in combating this parasitic infection. Helper T cells are important in initiation the activation of B cells in response to the gp90 foreign parasite protein, eventually leaving to active antibody secreting plasma cells. Flow cytometry will be useful in quantifying the number of immune cells present based on their different surface markers. Fluorochromes attached to specific marker antibodies will emit wavelengths that can be detected when the vaccinated blood sample is passed through the cytometer detector. All red blood cells must be removed from the blood sample in order to isolate the plasma portion of the blood. A Fluorochrome-labeled antibody specific for CD4 and CD8 cells is used. The cell suspension and antibody sample need to be incubated in a plate before they are run through the cytometer. A cell sorter activated by the fluorescence of the markers is used to separate the differing cells. The molecules can then be quantified and the results can indicate whether the vaccine produced a sufficient CD4 count necessary to initiate cellular immunity. A negative control is required and necessary to measure the adherence strength of the antibody. This can be done by including the antibody and isotype of the same species but using a different antigen.

Vaccine Proposal

Trypanosoma Cruzi is a parasite that infects millions of people in Latin America each year. Chagas Disease occurs when the parasite infects the red blood cells of an individual. This disease can be life threatening if it develops into the chronic stages. In the acute stages of the disease B and T cells help to control the infection. However, the parasite

will continue to live in the individual's system for the rest of their life. The chronic stages of the disease cause inflammation in the heart or the nervous system. The current treatment for the parasite are two oral drugs called Nifurtimox (4-(5-nitro-furylidenoamine)-tetrahydro-4-4-1,4-thiazine-1-1-dioxide) and o-benznidazole (N-benzyl-2-nitroimidazolacetamide) (Teixeira, 2006). The current drugs reduce the parasite load within the body; however, the parasite still remains in the host. The drugs also have a severe negative affect on the rest of the body.

Once the parasite has invaded the cell, a surface protein called gp90 is presented by MHC to the immune system (Andrew, 1995). Therefore, a vaccine needs to be produced that contains gp90 in order to create an immune memory to the parasite before the host is exposed to it. This can be administered to the host through an injection into the person's musculature. The vaccine itself would contain gp90 and aluminum hydroxide, which is an adjuvant. Once the vaccine has been injected it is engulfed by the dendritic cells and taken to the lymphnodes. Antigen will then be presented to the helper T-cells so they can then activate B-cells and create immune memory cells.

B-cells will then go through isotype switching from IgM to IgG. IgG is the primary antibody for use against intracellular parasites and is the most abundance class of immunoglobulins. When the parasite is present, the IgG will bind to the gp90 presented by MHC. Once bound, IgG will prevent the parasite from entering host cells because gp90 is required for cellular penetration and is blocked by the specific antibody. This results in a faster immune response to the parasite should a person become infected.

Our vaccine will consist of gp90 contained within a lipid cell called a micelle. The lipid compartmentalization is known as the iscom technique. By injecting this vaccine, the lipid micelle will fuse to the dendritic cell membrane and infuse the cell with the antigen gp90. The dendritic cell will then present the antigen on MHCI to present to CD8 cells. The induced immune response is discussed in further detail in the following sections.

The possible problem with this proposed vaccine is the fact there are several different alleles for the gp90 protein so vaccination against all forms is difficult. If our vaccine is proved ineffective, we could try using another antigen such as Cruzipain.

Ag163B6/Cruzipain is expressed abundantly throughout the parasites life cycle and immunization with this antigen can increase cytokine secretion of IL4, IL5, and IL10 (Savino et al, 2007). A vaccine using this antigen also seems very promising and is a good alternative if antigenic variation becomes a problem with gp90. Cruzipain in combination with a synthetic oligodeoxynucleotide containing CpG motifs was shown in a study to produce high specific antibody titers mostly composed of IgG2a. Spleen cells from the vaccinated mice showed a significant increase in IL2 and INF-gamma once stimulated by antigen, and elicited a TH1 immune response. The multicomponent vaccine could possible prevent new infection and eliminate tissue parasites preventing the autoimmune reactions of the host resulting in the chronic form of the disease (Frank et al, 2003).

Innate Response

The innate response is very important in the infection because a good response can prevent the devastating chronic effects that follow. When the host is inoculated with the parasite, complement is automatically activated which opsonizes the pathogen and aids in phagocytosis. Toll like receptors recognize the foreign antigen and trigger activation of the innate and adaptive immune responses. The inflammatory cytokine IFN γ activates macrophages along the classical pathway. With the aid of *T. cruzi* specific antibodies produced by the vaccine, complement is able to better bind antigen. Macrophages engulf the parasite while it is in the bloodstream and release cytokines such as TNF- α , and IL2 which activate IFN γ production by NK cells, recruiting leukocytes to the site of infection, and causing inflammation. The combination of IFN γ and TNF α leads to the production of NO which controls the levels of parasite growth (Martins, et al 2004). Cytokines such as IL4 and IL13 activate macrophages in an alternative pathway characterized by low production of inflammatory cytokines and involvement in type II responses. Chemokines such as CCL3, CCL4, and CCL5 also induce NO production to kill the pathogen (Savino et al, 2007).

Macrophage activation is not a very effective immune response due to the parasite's ability to escape the phagosome before lysosome fusion. NK cells possess strong cytotoxic capability against *T. cruzi* including the release of superoxides and other killing factors.

Adaptive Response

The decrease in apoptosis rates due to preliminary exposure to the gp90 antigen would have several effects on the adaptive immune response. The decrease in apoptosis would allow more build up of lymphocytes, increasing the total numbers of B cells and T cells in the lymph nodes and spleen. These cells would include CD8, CD4, and memory B cells. The CD28 marker on the surface of T cells is important in the activation of T cells and interferon gamma production by both CD4 and CD8 T cells in the *Trypanosoma cruzi* infection (Martins, et al. 2004). Without CD8 the infected individual is unable to produce NO to mediate parasite killing. The lymphocytes, with their extended life spans, can act as effector cells, enhancing the protective immune response. If the lymphocytes with specificity to the parasite were to undergo apoptosis at a high rate, it would be impossible for the immune system to develop a strong immune response.

Another possible mechanism for increased immune response is the use of a caspase inhibitor involving the role apoptotic cells play in advancing the infection. It has been shown that phagocytosis of apoptotic cells increases the growth of the parasite in infected macrophages (Silva et al, 2007). Blocking the apoptosis of these cells and decreasing the number of apoptotic cells the macrophages ingest may counteract the negative effects the parasite has on the immune system, and therefore act to stop immune suppression.

The free floating gp90 protein contained in the vaccine will activate gp90 specific B cells by binding to the antibodies present on their cell surface. The immunoglobulin, with

attached antigen, is then taken inside the cell and presented on class II MHC. A T_H2 cell that is specific for gp90 will then bind to the B cell MHC and signal it to become active through a combination of signals including CD40L and IL-4. The B cell then undergoes cell division and hypermutation, eventually producing plasma cells that secrete IgG specific for gp90. These antibodies will bind to the gp90 on any pathogen present, both inhibiting its ability to enter host cells and opsonizing it for ingestion by macrophages.

The gp90 contained in iscom lipid micelles will be absorbed into dendritic cells and presented on MHC, which will then be taken to the lymph nodes. Here the T cells will bind to antigen on dendritic cell MHC along with co-stimulatory molecules on the dendritic cell surface, causing them to become activated and secrete IL-2 along with the IL-2 receptor, causing it to proliferate. Activated T cells then leave the lymphoid tissues and find their way to the infection site, through both capillary adhesion molecules and chemotactic signals released near the infection.

The CD8 T cells, critical for controlling the *t. cruzi* infection, act by inducing apoptosis in the infected cells. They do this by recognizing gp90 peptide bound class I MHC on infected cells, and then killing them using a combination of enzymes held in lytic granules. These enzymes include perforin, an enzyme that creates pores in the membrane of the target cell, and granzymes, which are serine protease that activate the apoptosis pathway in the target cell. This controlled cell death releases any pathogen within the cell, where it is vulnerable to macrophages and antibodies.

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