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The Impact of Elevated Environmental Temperature on Cytokine Synthesis and Nitric Oxide Production During Experimental Chagas' Disease

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Honors Thesis
Directed by Dr. Cheryl D. Davis

Approved by

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May 3, 2000

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Abstract

C3H mice that have been infected with a Brazil strain of *Trypanosoma cruzi* and maintained at an elevated environmental temperature of 36°C survive an otherwise lethal infection. These mice show increased longevity and a dramatic decrease in parasitemia levels. In contrast, C3H mice maintained at room temperature typically experience high parasitemia levels and die within 40 days of infection. Previous studies in the laboratory suggest cell-mediated immune responses rather than antibody-mediated responses are responsible for the enhanced protection. The goal of the present study was to analyze cytokine synthesis and nitric oxide production in spleen cells from mice infected with 10^4 blood-form trypomastigotes of *Trypanosoma cruzi* and maintained either at room temperature or 36°C for 36 days. On day 36 of infection, mice were anesthetized. Blood was obtained by a cardiac puncture, and spleens were removed aseptically. A single cell suspension was prepared, and cells were diluted to a final concentration of 6×10^6 SC/ml. Spleen cells were incubated with or without concavalin A for 60 hours. Culture supernatants were analyzed for nitric oxide production using the Griess reaction; IFN-γ and IL-10 synthesis were measured by an antigen-capture ELISA. Results showed culture supernatants obtained from room-temperature mice had higher levels of nitric oxide and IFN-γ than mice maintained at 36°C. Levels of IL-10 were similar in both groups.

Introduction

Carlos Chagas: The Man, The Parasite and The Disease

Trypanosoma cruzi, the etiological agent of American Trypanosomiasis or Chagas' disease, is the obligate intracellular protozoan parasite that infects 18 – 20 million people in endemic areas of Latin America (W.H.O., 1991) and places approximately 100 million people at risk (see Figure 1) (W.H.O., 2000). Among those infected, approximately 50,000 will die each year because of the lack of any effective form of drug treatment and the absence of a vaccine (C.D.C., 2000). According to Tarleton et al. (1997), Chagas' disease is thought to be the single most common cause of congestive heart failure in the world and the leading cause of death in young to middle age adults in endemic areas of Latin America. The flagellated protozoan is the major public health problem for Latin American development because of its debilitating and lethal effects on the area's young adults (Bastien, 1998). Chagas' disease, more than any other parasitic disease of humans, is directly related to social and economic conditions of the areas it affects and will continue to undermine progression in Latin America so long as poor housing, frequent migration of people, and rapid urbanization persists as a way of life (W.H.O., 1991).

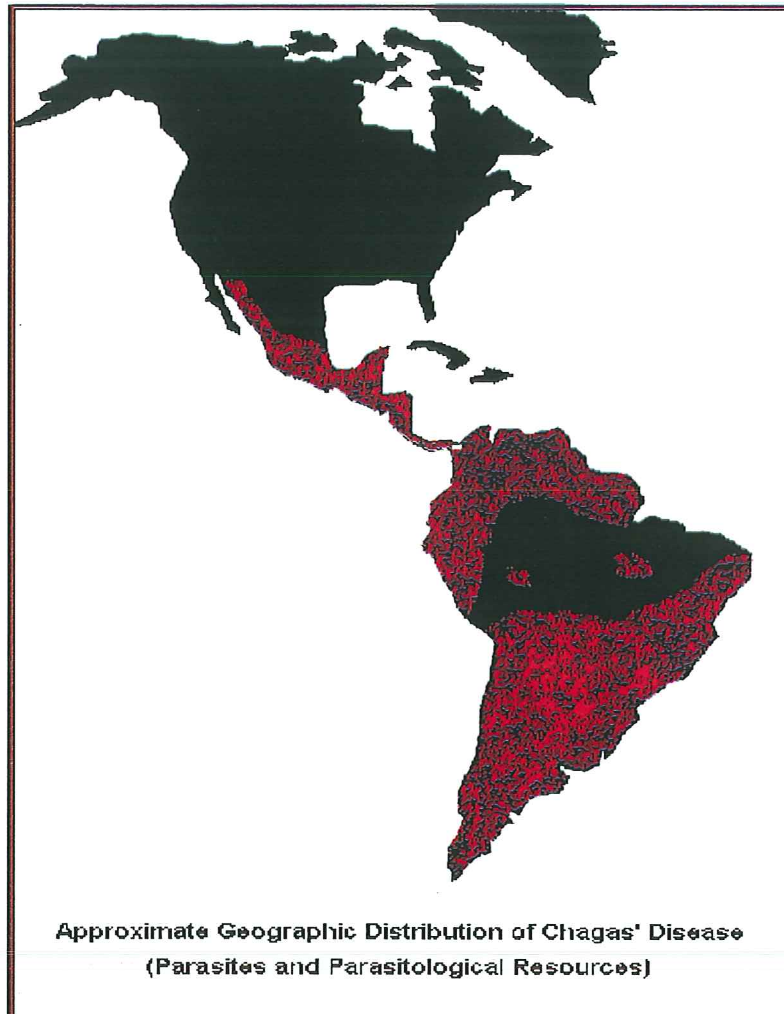

Figure 1. Geographic Distribution of Chagas' Disease
Source: Parasites and Parasitological Resources
http://www.biosci.ohio-state.edu/`parasite/home.html

Recent evidence suggests that the parasite has been on the South American continent in the region of present-day Peru and Chile for thousands of years. In 1997, a team of researchers from the Parasitology Center at the University of the Andes in Bogota, Columbia, found *T. cruzi* DNA in eight mummified bodies from the Atacama Desert (Guhl and Jaramillo, 1997). In 1985, anthropologists Rothhammer, Allison, Nunez, Standen, and Arriaza discovered the ancient mummies of twenty-two Andeans in Quebrada de Tarapaca, Chile. The mummies were approximately 1,500 years old and belonged to an extinct culture called the Wankuri. Eleven of the bodies had greatly enlarged hearts, colons, or esophagi that are characteristic of chronic infection with *T. cruzi*. The exhumed Wankuri likely died from Chagas' disease, which was possibly as debilitating and deadly as it is now (Bastien, 1998).

Trypanosoma cruzi, the small protozoan that had ravaged Latin America for thousands of years, largely went unnoticed until the beginning of the twentieth century with the work of one man – Dr. Carlos Justininiano Roberio Chagas (see Figure 2). Born July 9, 1879, in the small town of Oliveira, Minas Gerais, Brazil, Chagas was the son of Portuguese farmers who were descendents of immigrants that had come to Brazil in the late seventeenth century. His upper-class parents owned a small coffee plantation that generated a modest income. Chagas' father died when he was only four years old and his mother, a strong-willed farmer, raised him and his three siblings. His mother urged the young Brazilian to become a mining engineer, but Chagas refused and instead opted for medical school at the advice of his physician uncle, Carlos Ribeiro de Castro, who convinced him that for the struggling nation of Brazil to develop industrially it was necessary to rid the country's landscape of endemic diseases that commonly plagued it.

Figure 2. Carlos Chagas in front of the Oswaldo Cruz Institute
Source: Biblioteca Virtual Carlos Chagas
http://www.prossiga.br/chagas/

In fact, many European ships refused to dock in Brazilian ports because of the fear of contracting yellow fever, smallpox, bubonic plague, and syphilis (Bastien, 1998).

Carlos Chagas studied at the Manguinhos Institute in 1902, where he wrote his M.D. thesis on the "Hematological Aspects of Malaria" (1903) under the leading Brazilian parasitologist, Oswaldo Cruz. Cruz had successfully tackled the task of ridding Rio de Janeiro of yellow fever by systematically combating the *Anopheles* mosquito vector and isolating victims in special hospitals. Both measures were revolutionary at that time. When Cruz invited Chagas to work on his malaria research, Chagas refused, stating that he was not prepared to do the research and preferred to practice family medicine instead. Chagas worked in a hospital at Jurujuba from 1903 until 1905, where he introduced anitpest serotherapy, which Cruz had modified from methods introduced by Louis Pasteur in France around 1890. Following Pasteur's and Cruz's assumptions that negative organic elements fermented positive organic elements, Chagas first prepared an antiseptic serum, then cut into a patient's swollen glands and inserted this serum to destroy the malarial parasite. Chagas proved to be a very innovative and experimental physician who looked for answers in practice instead of in the laboratory (Bastien, 1998).

On March 30, 1905, the Santos Dock Company of Santos, hired Chagas to combat the *Plasmodium* parasite, the etiological agent of malaria transmitted by the female *Anopheles sp.* mosquito vector. The company's workers were so weakened by the disease that they could not complete the port of Santos, the most important seaport in Brazil and one which would permit coffee exportation from the city of Sao Paulo (Bastien, 1998). Although no long-lasting insecticides such as DDT were available at this time, Chagas focused his efforts on destroying the mosquito vector that harbored the

deadly parasite (Wendel and Brener, 1992). Chagas developed a threefold program in Santos that became the standard for malaria campaigns in other regions of Brazil by 1917. The first step consisted of quinine dosages of 50 centigrams every three days to infected patients. These patients were also isolated in infirmaries with fine metal screens. Additionally, quinine was administered to other people in the region who had begun to show malarial symptoms. Finally, domiciles were periodically and systematically disinfected with pyrethrum, which killed the mosquitoes in flight. Chagas further contributed to the field by describing the edematous form of Quartan fever and the bone-marrow lesions of malaria. He was also the first to describe malaria as a domiciliary infection that was rarely contracted outdoors (Bastien, 1998).

In 1908, the Brazilian government was attempting to link Belem, located near the mouth of the Amazon River, with Rio de Janeiro, but construction was halted in Minas Gerais because of a severe malarial attack that was killing thousands of railroad workers. Oswaldo Cruz commissioned Carlos Chagas and Belisario Pena to that region, where they settled in Lassance, making their headquarters inside a railroad car - - which served as a consultation room, a laboratory, and their sleeping quarters. After one year of grueling work, a railroad engineer advised Chagas about the existence of hematophagus bugs, which were known as "barbeiros," "kissing bugs," or "vinchuca" (from the family *Reduvidae*) because of their atypical behavior of biting humans on their faces while they were sleeping at night. Chagas then became interested in researching the possibility that these bugs were transmitting parasites to humans or other vertebrates (Wendel and Brener, 1992). Chagas observed that the bugs were sensitive to light: during the daytime they would typically hide in cracks and crevices of walls and ceilings where they would

rest, copulate, and lay eggs. Barbeiros are considered vampire bugs because they become active at night, descend from their nests, and draw blood from animals, including humans (Bastien, 1998).

After dissecting and examining the bugs for parasites, Chagas detected flagellates resembling crithidae in the bugs' hindguts. Chagas was intrigued by the possibility that the parasites could represent another stage of *Trypanosoma minasense*, which he had previously described in infected marmosets in 1908. He sent some of the reduviid bugs to Manguinhos to feed on primates that were free of infection. After several weeks, the same flagellates that Chagas had observed in the field were recovered in the bloodstream of the infected primates. Chagas had discovered a new species, different from *Trypanosoma minasense* or any other species of the same genus. The parasite was first given the name *Schyzotrypanum cruzi*, in honor of Oswaldo Cruz, but was subsequently renamed *Trypanosoma cruzi* (Wendel and Brener, 1992).

Chagas returned to Lassance in the spring of 1909 looking for the presence of vertebrate hosts of this newly discovered trypanosome, and after several tests on humans and other animals, he was able to find a cat with the parasite circulating throughout its bloodstream. Two or three weeks later, Chagas was asked to investigate the possibility of an acute phase malarial infection in a two-year-old girl named Berenice, who was living in the same house where the infected feline was discovered. He had previously examined the girl but detected no parasites in her bloodstream at that time. "Therefore, he suggested the possibility of an acute phase infection of a disease yet to be described" (Wendel and Brener, 1992). Several examinations showed the disappearance of flagellates in the bloodstream as the symptoms vanished, thus raising the possibility of a

chronic phase of infection for this new disease, an infection that had moved from the blood and lymph to body tissues (Wendel and Brener, 1992).

On April 23, 1909, Oswaldo Cruz announced Carlos Chagas' discovery at a session of the Brazilian National Academy of Medicine. In August of 1909, Chagas published his findings in the first volume of Memorias do Instituto Oswaldo Cruz. In his paper, "New Human Trypanosomiasis," Chagas described the human infection, the parasite morphology in the bloodstream, the parasite's life cycle in the digestive tract of the invertebrate vector, the cultivation of the parasite in agar-blood, and the transmission of flagellates to vertebrates via infected reduviid bugs. Although some slight errors were made in relation to the parasite's life cycle- - mainly Chagas' idea that the parasite was transmitted in the saliva of the insect and not the feces- - the great contribution of this work clearly surpassed any minor imperfections (Wendel and Brener, 1992).

Chagas, while in Lassance in 1909, also stumbled onto another parasitic organism that formed peculiar cysts in the lungs of guinea pigs infected with *Trypanosoma cruzi*. He proposed that these organisms were schizonts that belonged to the life cycle of the protozoan parasite, thus redefining the genus as *Schyzotrypanum*. Chagas' work also agreed with the work of Antonio Carinii, who published similar descriptions of cysts in the lungs of rats infected with *Trypanosoma lewisi*. It was not until a French husband and wife team, the Delanoes, reviewed Carinii's histological slides and research papers and conducted experiments on Parisian sewer rats that these cysts were actually determined to be separate organisms unrelated to the trypanosomes. They named this new organism *Pneumocystis carinii* and after this discovery both Chagas and Carinii retracted their original findings and subsequently provided data that supported their

research. *Pneumocystis carinii* is of great importance today because it is the number one parasitic infection of AIDS and other immunocomprimised patients (Armengol, 1995).

In 1911, Chagas presented at the National Academy of Medicine in Rio de Janeiro the first report of a congenital case of what came to be known as Chagas' disease, and in 1912, he reported the possibility of a sylvatic cycle in armadillos. In 1916, he suggested that the digestive tract could be involved, especially related to megaesophagus and dysphagia, maladies which had been regionally known for over a hundred years. After the death of Oswaldo Cruz in 1917, Chagas replaced the famed Brazilian as the director of the Manguinhos Institute, a position he held until his death, and confronted the difficult task of controlling Spanish Fever in Rio de Janeiro (Wendel and Brener, 1992). "Carlos Chagas died in 1933 of angina pectoris as he was looking through a microscope into the universe of parasites" (Bastien, 1998). A year before his death he remarked, "This is a beautiful land, with its tremendous variety of vegetation. Nature made animal and vegetable life stronger and thus created conditions which bring sickness and death to the men that live here" (Bastien, 1998).

Life Cycle of the Parasite

When reduviid bugs feed on mammalian hosts, they often defecate on the skin. Metacyclic trypomastigotes may be present in the insect's feces and can gain entry into the vertebrate host's body through the bite, through scratched skin, or through mucous membranes that are rubbed with the host's fingers which have been contaminated by the insect's feces. Reservoir mammalian hosts are infected when they consume infected reduviid bugs. Although numerous blood-form trypomastigotes (BFT) are present in the

host's circulation during early infections, they do not reproduce until they invade a host cell and transform into the amastigote stage. Reticuloendothelial cells of the liver, spleen, and lymphatics are frequently invaded, as are cells in cardiac, smooth, and skeletal muscle tissues. The nervous system, skin, gonads, intestinal mucosa, bone marrow, and placenta may also become infected in some cases (Roberts and Janovy, 1996).

Soon after entering the host cell, the parasite loses its undulating membrane and flagellum and begins to reproduce at a rapid rate. "Repeated binary fission produces so many amastigotes that the host cell is killed and lysed" (Roberts and Janovy, 1996). After cell death and subsequent parasite release, the protozoa begin to attack other host cells. Pseudocysts (cystlike pockets of parasites) form in muscle cells (Roberts and Janovy, 1996).

Trypomastigotes are ingested by reduviid bugs and then pass through the posterior portion of the insect to the midgut, where they transform into short epimastigotes that multiply by longitudinal division to become long, slender epimastigotes. Approximately eight to ten days post infection, metacyclic trypomastigotes can be found in the insect's rectum and may infect an unsuspecting mammalian host. Release of the trypomastigote stage into the patient's bloodstream causes onset of the acute phase of infection (Roberts and Janovy, 1996).

Chagas' Disease Pathology

Chagas' disease manifests acute, intermediate, and chronic phases. The acute phase of infection typically begins one to three weeks post infection and is most common

and severe in children less than five years old (Roberts and Janovy, 1996). Acute Chagas' disease is usually a mild illness with fatality rates less that five percent (Kirchhoff, 1993). After trypomastigote stages enter the wound site, a local inflammation- - termed a *chagoma* and caused by the swelling of regional lymph nodes- - is outwardly visible. In approximately fifty percent of all cases, trypomastigotes enter through the conjunctiva of the eye and produce a clinical symptom known as Romana's sign (Roberts and Janovy, 1996). General symptoms associated with the acute phase include fever, malaise, edema of the face and lower extremities, generalized lymphadenopathy, and heptasplenomegaly. Muscles are parasitized heavily, including cardiac tissue. Severe myocarditis may develop in a small number of cases. The acute phase will last over a period of four to six weeks in most individuals who will subsequently enter the asymptotic intermediate phase of infection (Kirchhoff, 1993).

During the intermediate phase of infection, patients will typically have low-grade parasitemias in conjunction with antibodies to many *T. cruzi* antigens. Most Chagasic patients will remain in the intermediate phase for life, and it is in this manner that the scene is set for transfusion-associated transmission of *T. cruzi* (Kirchhoff, 1993). Nearly 10 to 30 years post infection, 30% of patients will enter a chronic phase of the disease in which they develop a severe cardiomyopathy which is responsible for the majority of annual deaths attributed to the parasite (Wizel et al., 1997). Cardiac tissue is heavily infected in the majority of chronic cases, resulting in biventricular enlargement, thinning of ventricular walls, apical aneurysms, and mural thrombi. "The conduction system is often affected, typically resulting in right bundle-branch block, or complete atrioventricular block" (Kirchhoff, 1993). Death results primarily from congestive heart

failure or disturbances in heart rhythm. Some Chagasic patients develop megaesophagus, megacolon, or a combination of the two referred to as megadisease which is associated with dysphagia, regurgitation, repeated aspiration, and severe constipation (Kirchhoff, 1993).

Diagnosis and Treatment

The acute phase of *T. cruzi* infection is diagnosed by the detection of parasites circulating in the blood of the patient. These extremely dynamic forms can be easily seen during microscopic examination of anticoagulated blood or buffy coat. The parasite is also visible in stained smears. Another method that is widely used is xenodiagnosis. Diagnosis of the parasite is made upon the examination of laboratory reared reduviid bugs that are allowed to feed upon the patient. This technique can detect cases in which concentrations of circulating trypomastigotes are too few to be found by ordinary diagnostic means (Roberts and Janovy, 1996).

Complement fixation and other immunodiagnostic tests are extremely effective in diagnosing chronic cases. However, these tests may give false positive reactions if a patient is infected with *Leishmania sp.* or another species of trypanosome. Indirect fluorescent antibody tests (IFAT), enzyme-linked immunosorbent assay (ELISA), and dot-blotting immunobinding assays have all been used in diagnosing chronic cases in laboratory studies. Diagnostic methods based on the detection of parasite DNA that utilize the polymerase chain reaction (PCR) have been developed, but have not been widely used because of cost and problems with false negatives (Roberts and Janovy, 1996).

Unlike other trypanosomes that infect humans (*T. brucei brucei, T. brucei gambienese*, and *T. brucei rhodesiense*), *Trypanosoma cruzi* does not respond well to any kind of chemotherapeutic agents, and there is no vaccine available for use at this time. The most effective drugs kill the extracellular stages of the parasite, leaving the host vulnerable to the development of the intracellular stage (Roberts and Janovy, 1996).

Transmission

As a result of rapid rural migrations to urban areas in the 1970s and 1980s, traditional epidemiological patterns of Chagas' disease were forever changed, as it became an urban disease with unscreened blood transfusions serving as an alternative mode of transmission. According to the most recent statistics provided by the World Health Organization (2000), between 1960 and 1989, the prevalence of infected blood in blood banks in selected cities of South America ranged from 1.7% in Sao Paulo, Brazil, to 53.0% in Santa Cruz, Bolivia, a percentage far higher than that of hepatitis or HIV infection. Transmission from mother to infant through breast milk may be possible, but the parasite may cross the placental membrane, infecting the developing fetus (Roberts and Janovy, 1996).

Because of the rapid influx of Latin American immigrants to the United States, infections due to blood transfusions are our biggest threat. In Southern California alone, forty percent of all blood donors are Latin Americans and presently blood banks are not mandated to test for *T. cruzi* because no commercial screening has been licensed by the Federal Drug Administration (Wendel and Gonzaga, 1993). An associated risk of *T. cruzi* infected immigrants is the risk of parasite transmission by transplantation of their

donated organs. A host of reports from endemic areas of Latin America describe transmission of the disease by the transplantation of infected kidneys to donors. "Although the probability of such transmission is not known, it can be presumed to be substantial" (Kirchhoff, 1993).

Interferon-γ Synthesis during *Trypanosoma cruzi* Infections

Interferon-γ is a T_h1 cytokine that is produced by come CD4+ T-helper lymphocytes and almost all CD8+ cytotoxic T lymphocytes. It is a very potent macrophage-activating factor, causes a variety of cells to express class II MHC molecules, promotes differentiation of T and B cells, activates neutrophils and NK cells, and activates endothelial cells to allow lymphocytes to pass through the walls of blood vessels (Roberts and Janovy, 1996). A number of studies have shown INF-γ synthesis is crucial in the control of *T. cruzi* infection, especially during the acute phase. A study conducted in 1991 by McCabe and colleagues examined the effects of recombinant murine IFN-γ (rmuIFN-γ) administered to cyclosporin-treated mice with either acute or chronic *T. cruzi* infection. Their results indicated that treatment with rmuIFN-γ in cyclosporin-treated mice with an acute infection resulted in highly suppressed parasitemia levels resembling those observed in immunocompetent mice. They also found that rmuIFN-γ prevented death due to *T. cruzi* infection in immunocompromised mice when the innoculum of parasites was relatively small. However, no beneficial aspects were observed in cyclosporin-treated mice suffering from a chronic *T. cruzi* infection (McCabe et al., 1991).

In another study, investigators examined the role of endogenous IFN-γ in resistance to acute infection in BALBc/J mice that were injected with a potent anti-IFN-γ monoclonal antibody. Results indicated that treatment with the neutralizing antibody increased parasite burden in the blood and in the tissues and considerably shortened survival time. Although previous studies had shown INF–γ and parasite-specific antibodies working synergistically to confer protection to infected mice, they found that IFN-γ-mediated protection is evident when antibody levels are very low. They proposed that IFN–γ controls the first step of parasite multiplication by activating effector cells, "allowing mice to survive and to mount a humoral and cellular immune response, which, in a second phase, eliminates the remaining parasite population" (Torrico et al., 1991). Their findings also suggest that CD8+ T cells and/or natural killer (NK) cells are the main producers of IFN–γ during the acute phase of infection (Torrico et al., 1991).

Interleukin-10 Synthesis during *Trypanosoma cruzi* Infections

Interleukin 10 (IL-10) is a T_h2 cytokine derived from CD4+ T-helper cells. Its synthesis and activity indirectly inhibits the secretion of cytokines by T_h1 T-helper lymphocytes, CD8+ cytotoxic T lymphocytes, natural killer cells (NK) and macrophages (Roberts and Janovy, 1996). IL-10 was first described in studies that showed its antagonistic effects on the production of IFN-γ by T_h1 CD4+ T cell clones. Later studies concluded that this activity was indirect and was due to the ability of IL-10 to block the synthesis of tumor necrosis factor – α (TNF-α), interleukin 1 (IL-1), and interleukin 12 (IL-12)- - cytokines which were needed for optimal stimulation of IFN-γ synthesis by T cells- - in macrophages. IL-10 also can limit the expression of class I and class II major

histocompatibility complex (MHC) molecules and B7 costimulatory molecules on the surface of macrophages. Further studies showed that IL-10 antagonizes the ability of IFN-γ to activate the antimicrobial effector mechanisms of macrophages. Thus, when all data are considered together, it became clear that synthesis of IL-10 down-regulates many of the factors required for the establishment of a cell-mediated immune response (Hunter et al., 1997).

A study conducted in 1997 by Hunter et al. utilized IL-10 knockout (KO) mice infected with a virulent strain of *T. cruzi*. Their results showed that IL-10 mutant mice experienced greatly reduced parasitemia levels and reduced numbers of parasites in heart tissue when compared with normal wild type mice. However, the IL-10 KO mice succumbed to infection earlier than wild type mice. The authors suggested that while production of IL-10 may result in an increased parasite burden, the cytokine is required to prevent a pathogenic CD4+ T cell response associated with the overproduction of IL-12 and IFN-γ. The study also demonstrated that NK cells were the major source of IFN-γ in infected IL-10 KO mice. Their data also revealed that other immunosuppressive cytokines (TGF-β, IL-4 and IL-13) did not compensate for the loss of IL-10 activity in this system. The researchers were able to conclude that since IFN-γ synthesis in T cells is only apparent in the absence of IL-10, the cytokine is vital in maintaining the delicate equilibrium between a pathogenic response and a protective immune response during Chagas' disease.

In a more recent study conducted in 2000 by Abrahamsohn and colleagues, it was shown that treatment with recombinant IL-4 and recombinant IL-10 in 129/J mice leads to a marked increase in parasitemia levels and a drastic decrease in IFN-γ synthesis in

stimulated spleen cells. Besides decreasing the secretion of IFN-γ, the two cytokines can directly neutralize IFN-γ-mediated macrophage anti-trypanosomal activity and enhance parasite resistance and survival. The study also concluded that IL-4 was not a major regulator of IFN-γ synthesis or a "determinant of susceptibility in the acute phase of Y strain *T. cruzi* infection" (Abrahamsohn et al., 2000). On the hand, IL-10 was shown to be much more important at limiting resistance to infection (Abrahamsohn et al., 2000).

The Role of Nitric Oxide in the Immune Response

At the crossroads of the innate and adoptive immune systems lies NOS2 (iNOS), the high-output isoform of nitric oxide synthase that requires at least 17 binding reactions to assemble a functional dimer. NOS2 was the first isoform cloned from macrophages and is the most widely distributed among cell types. All three mammalian isoforms of NOS (NOS1, NOS2 and NOS3) are believed to catalyze NO production by the same biochemical pathway, consisting of two sequential monooxygenase reactions in which one molecule of L-arginine is oxidized at a guanidino nitrogen to produce N ω–OH–L-arginine as an intermediate and is further oxidized to yield one molecule each of NO and L-citrulline (MacMicking et al., 1997).

A host of cytokines and microbial products, often acting synergistically in pairs, stimulate expression of NOS2. "The effective agents and combinations depend on cell type and species" (MacMicking et al., 1997). With inflammatory mouse peritoneal macrophages, IFN-γ has been the only cytokine shown effective when tested alone. The synergy afforded by the combination of TNF with any of the interferons is particularly important because the intake of a host of microbes elicits autocrine production and action

of both TNF and IFNα/β. "The physiological relevance of IFN-γ as an inducer of NOS2 is evident in the difficulty with which NOS2 is expressed in the macrophages of mice rendered deficient in IFN-γ or its receptor" (MacMicking et al., 1997). On the other hand, NOS2 expression is suppressed by the actions of IL-10, IL-4, TGFβ1-3, IL-13, osteopontin, α-melanocyte-stimulating hormone, taurine chloramine, 2,4-diamine-6-hydroxypyridine, macrophage stimulatory protein 1, and NO itself (MacMicking et al., 1997).

In cell lines and inbred mice strains, resistance to microbial growth is often associated with expression of NOS2, and NOS inhibitors have been shown to worsen the course of infection caused by a wide variety of microbial pathogens- - including viruses, bacteria, fungi, protozoa, and helminths. Monocytes and macrophages also produce O_2^-, H_2O_2, $^{\cdot}OH$ and occasionally HOCL in many of the same conditions that they produce NO. These agents may inactivate NO or convert it into an even more potent antimicrobial product. On the other hand, they could act synergistically with NO to produce a more powerful effect (MacMicking et al., 1997).

The antimicrobial view of NO production is complicated by the ability of the same pathway to suppress harmful immune responses, suppress beneficial immune responses, and damage host tissues. A large amount of NO may inhibit cytokine secretion, but smaller amounts of NO may induce cytokine synthesis. Antigen presenting cells also are affected by NO production, and high-output production of NO can suppress transcriptional activation of several genes, including NOS2. In some settings, macrophages seem to trigger their own cell death through NO production, but this is not observed uniformly (MacMicking et al., 1997).

As potent as NO is to microbes, it is also very deleterious to host cells. However, the utility of NO as an antimicrobial agent is adequately high enough that despite the ensuing risk of damage to host cells, most types of nucleated cells are programmed to make it in large amounts when stimulated by inflammatory cytokines and microbial products. A noted effect of NO production is the fact that the high-output path of NO production can contribute to bacterial septic shock and kill the host just as surely as the mounting numbers of microorganisms (MacMicking et al., 1997).

Nitric Oxide Production in *Trypanosoma cruzi* Infections

Several studies have analyzed the efficacy of NO production in controlling *T. cruzi* infections in murine models. One study analyzed the efficiency of immature macrophages to contain the parasite as compared to mature macrophages. A study conducted by Plasman et al. in 1994 showed that immature murine peritoneal macrophages (MPM) were more susceptible to *T. cruzi* infection and their α_2-macroglobulin (a proteinase inhibitor) production was considerably lowered. Their findings also showed that IFN-γ activated immature MPM produced lower levels of NO and TNF-α levels. Thus, as a direct consequence of leukocyte maturation, immature MPM were more susceptible to infection than mature MPM, which had higher levels of α_2-macroglobulin, NO and TNF-α production (Plasman et al., 1994).

Several studies have addressed the role of NO inhibitors on *T. cruzi* infections. One study conducted by Vespa et al. in 1994 showed that splenocytes obtained during the acute phase of infection contained elevated levels of NO which corresponded with host resistance or decreased susceptibility, while inhibition of NO production resulted in

increased parasitemia levels and mortality. Their results also showed that the NO donor drug S-nitro-acetyl-penicillamine is able to kill the circulating trypomastigote stage *in vitro* in the absence of any other cell line, suggesting a direct NO-mediated killing of the parasite (Vespa et al., 1994). These findings also were substantiated by similar findings from Petray and his colleagues that same year (Petray et al., 1994).

In a more recent study conducted in 1998 by Holscher et al., *T. cruzi* infection in mice that were IFN-γ receptor and iNOS (NOS2) deficient was evaluated. Both strains of mutant mice showed relative susceptibility to parasitic infection and succumbed during the acute phase of infection even though both lines of mice were infected with relatively low numbers of trypomastigotes. Both mutant strains were unable to produce NO in response to infection with the parasite *in vivo* and *in vitro*. Macrophages from both mutant strains were defective in trypanocidal activities, and parasites replicated rapidly in these cells. Their results strongly suggest the crucial importance of iNOS mediated NO production as the major defense mechanism for surviving *T. cruzi* infection. IFN-γ dependent iNOS expression seems mandatory for surviving *T. cruzi* infection, and IFN-γ activation is required for the production of TNF-α and IL-1α in activated macrophages (Holscher et al., 1998).

In other reported studies, researchers also have found that IFN-γ plays a crucial role in parasite killing not only by the production of NO but by the expression of Fas and Fas-L independently of NO. Results indicated that besides modulating NO-induced apoptosis, IFN-γ could lead to apoptosis by mediating an enhancement in Fas and Fas-L expression. Fas is a type I membrane protein that belongs to the TNF/nerve growth factor receptor family and is expressed constitutively on the surface of activated B and T

lymphocytes, hepatocytes, and other tissues and tumors. On the other hand, Fas-L (CD95 ligand) is a type II transmembrane protein that is expressed in a host of tissues- - including the spleen, thymus, lung, testis, heart and small intestine. Prior investigations have implicated Fas expression in the induction of apoptosis in mice infected with another protozoan parasite, *Toxoplasma gondii*. However, this investigation also proved to be the first link established between Fas expression and apoptosis in a *T. cruzi* model. Results suggested that NO-mediated parasite killing and control of inflammation through apoptosis induction could be involved in limiting the damage to host tissues and promoting the establishment of the chronic disease in infected mice (Martins et al., 1999).

Other immunological molecules have been shown to increase NO production in Chagasic murine models. Recently there has been a significant amount of interest in chemokines, an innovative class of inflammatory mediators which appears to play a role in the mediation of extravasation and accumulation of specific leukocytes in acute and chronic inflammatory processes in several diseases. Chemokines are released from a wide assortment of different cell types after activation and have been shown to have a potent chemotactic activity both *in vitro* and *in vivo*. As well as having intense effects on the movement of leukocytes, chemokines appear to be affect several other biological events, including T lymphocyte proliferation, T_h1 and T_h2 differentiation, natural killer cell migration and activation, and macrophage production of IL-1 and IL-6. In 1999, Aliberti and colleagues examined whether Chagasic infection triggered beta-chemokine mRNA expression and protein production and if these chemokines were involved in the regulation of NO by infected murine macrophages. Their results showed that macrophage-derived chemokines drove NO production in activated macrophages, but

when chemokines were added to these infected cell lines, NO production was induced and intracellular growth of the parasite was reduced in a NO-dependent manner. They also observed that a specific beta-chemokine, JE/MCP-1, synergized with IFN-γ to control parasite replication *in vitro*.

Also in 1999, Aliberti and colleagues analyzed the impact of platelet activating factor on the production of NO during infection with *T. cruzi*. Platelet activating factor (PAF) is a membrane-derived phospholipid with widely accepted proinflammatory activities and is produced in an assortment of leukocytes, including neutrophils, eosinophils, lymphocytes, and macrophages. PAF has been implicated in several systemic and organ-specific disorders, but little is known about its role during infection with *T. cruzi*. Studies have shown a direct association between the ability of PAF to induce NO production and antimicrobial activity. Researchers also found that PAF potentiates macrophage anti-trypanosomal activity when added simultaneously with IFN-γ, an effect which suggests that PAF binding and signaling through a specific receptor leads to parasite killing by the NO pathway during the early stages of infection *in vitro*. This study suggests that PAF is produced during *T. cruzi* infection and together with parasite triggered proinflammatory cytokines (TNF-α, INF-γ and IL-12) mediates resistance to infection. In contrast, blocking of these cytokines or PAF leads to intensified parasitemias and early death. An associated issue concerns the role of PAF in acute myocarditis in mice infected with the parasite. Since PAF serves as a chemoattractant for several leukocyte populations, it is possible that trypomastigote stages present in the heart lead to PAF secretion, which in turn leads to inflammatory –

cell penetration and synthesis of inflammatory cytokines in cardiac tissue (Aliberti et al., 1999).

Nitric oxide production seems to be triggered by certain properties of the parasite itself. One such molecule identified is the glycosylphosphatidylinositol-anchored mucin-like glycoproteins (GPI-mucins) isolated from the surface of trypomastigotes, a molecule which is responsible for the initiation of cytokine synthesis by host macrophages. Camargo and colleagues (1997) were able to show that only vertebrate-derived host stages, trypomastigotes and amastigotes, were able to trigger synthesis of NO by macrophages primed with INF-γ. Most of the activity seemed to be associated with parasite membranes. Specifically, trypomastigote-derived GPI-mucins served as the major parasite component that triggered NO production by inflammatory macrophages. The study also revealed that it was GPI anchors from GPI-mucins that were directly involved in the triggering of NO production in macrophages stimulated with IFN-γ (Camargo et al., 1997).

Secretory antigens produced by the parasite also have been experimentally proven to induce NO synthesis. One such secretory antigen is Tc52 (Molecular mass: 52kDa), which is composed of two homologous domains which share significant homology to glutathione S-transferases. Fernandez-Gomez and colleagues (1998) have shown that Tc52 is expressed by intracellular amastigotes and is able to suppress human and murine T cell proliferation *in vitro*. Results of their studies indicate that Tc52 synergized with IFN-γ increased expression of iNOS mRNA and increased levels of NO production by macrophages. An unexpected effect of the secretory protein is the induction of genes encoding for IL-1α, IL-12 and IL-10. Studies also have demonstrated that Tc52 exerts a

regulatory effect on inflammatory and immune responses and may play a role in *T. cruzi*-associated immunosuppression (Fernandez-Gomez et al., 1998).

Elevated Environmental Temperature Phenomenon

A number of studies have confirmed the beneficial aspects of elevated environmental temperature in mice infected with *Trypanosoma cruzi* and other related trypanosome species. In 1989, Anderson and Kuhn performed studies to determine which aspects of the immune response were conferring protection to C3HeB/FeJ (C3H) mice infected with a Brazil strain of *T. cruzi* and maintained at an elevated environmental temperature. They examined alterations in the host immune system, the production of antibody to heterologous antigen, and the establishment of parasite-specific T_h cells in non-infected and infected C3H mice. As shown in previous studies, they found that mice maintained at 36°C are significantly more resistant to the parasite than those mice maintained at room temperature (20 to 24°C). Their findings also showed that the internal body temperature of mice maintained at 36°C increases approximately 3-4°C, which is similar to increases seen in a natural febrile response.

Mice were immunized with either sheep erythrocytes or trinitrophenyl groups (TNP) conjugated to fixed culture forms of the parasite. The splenic direct plaque-forming cell (DPFC) responses to sheep erythrocytes and to TNP-conjugated sheep erythrocytes were determined. The role of elevated environmental temperature on antibody production in mice immunized with sheep erythrocytes was measured and compared with responses in age-matched non-infected controls. Results showed that mice maintained at 36°C experienced a significantly more elevated anti-sheep erythrocyte

DPFC response than room temperature mice, and mice maintained at 36°C showed significant enhancement of parasite-specific responses of T_h cells (Anderson and Kuhn, 1989).

In 1991, Dimock et al. investigated the production of parasite-specific antibodies during the acute phase of *T. cruzi* infection in C3H mice maintained at 36°C and at room temperature. The results of the study showed that mice maintained at room temperature experienced very high parasitemias, developed high levels of parasite-specific antibodies, and a 100% mortality rate. However, mice maintained at 36°C experienced relatively low parasitemia levels, low levels of anti-*T. cruzi* antibodies, and a 100% survival rate. On day 35 of infection there were no parasite antigens recognized by sera from 36°C mice that were not recognized by sera from room temperature mice. The authors concluded that either parasite-specific antibodies do not play a significant role in the enhanced immunity or that the antibody response to low levels of antigen suggests their importance. They also suggested that low levels of antigenic stimulation may inhibit antibody formation early in the course of infection, and that some other aspect of the immune response must be responsible for the enhanced protection. Possibilities included the enhancement of cell-mediated responses such as the T-helper cell response or the production of cytokines such as interleukin 1 (IL-1) or interleukin 2 (IL-2). However, their results emphatically showed that survival of mice maintained at 36°C during the acute phase of infection could not be attributed to levels of parasite-specific antibodies alone.

In 1992, Dimock et al. designed an experiment to examine anti-*T. cruzi* antibody production throughout infection using a parasite-specific ELISA and immunoblot

analysis. Their findings showed an increase in the production of parasite-specific antibodies in addition to increases in the number of parasite antigens recognized throughout the course of infection in mice maintained at 36°C. Although they noted differences in antigens produced by culture forms at 27°C and 40°C, they found there was no significant difference in antigen recognition by sera from both groups of mice. However, a 61kDa-parasite antigen produced by mice maintained at 36°C was found to be strongly recognized by sera from mice maintained at 36°C, while sera from room temperature mice only faintly recognized that same antigen. Both groups of mice produced a 19-kDa antigen, but a more intense reaction was seen with sera obtained from 36°C mice. These results suggested either variable antigenic presentation or various immunogenic properties of antigens produced at the two temperatures, and provided evidence for a strong antibody response in mice maintained at 36°C.

A study conducted in by Arif et al. (1999) examined antibody response to heat shock proteins and histopathology in C3H mice infected with a Brazil strain of *T. cruzi* and maintained at 36°C. Results showed that mice maintained at 36°C experienced greatly reduced cardiac and skeletal muscle tissue pathology than what is typically associated with murine Chagasic models. Skeletal and cardiac tissue from room temperature mice analyzed on day 42 post infection (PI) showed extensive tissue necrosis with large numbers of pseudocysts and a heavy infiltration of mononuclear cells. However, cardiac tissue from mice maintained at 36°C analyzed on day 15 and day 25 PI showed no clinical evidence of host cell infiltration, tissue necrosis, or tissue-dwelling parasite forms. By day 35 PI, only a low number of pseudocysts and mild inflammation was observed in cardiac sections. No parasites were found in skeletal muscle tissue

throughout the course of infection in 36°C mice. The authors suggest that there is a direct correlation between the numbers of circulating BFT and the severity of tissue pathology and infection in the elevated environmental temperature model of *T. cruzi* infection.

To analyze the role of heat shock proteins (HSPs) in this model, an immunoprecipitation assay was performed to determine if parasite HSPs were acting as the primary targets of the humoral immune response in mice maintained at 36°C. Results showed that parasite HSPs represented major antigens precipitated by sera from infected 36°C mice but room-temperature mice also produced these same antigens. However, "anti-HSP antibody levels did increase over the course of infection in *T. cruzi*-infected mice maintained at 36°C and by day 45 PI were higher than observed in room temperature infected mice" (Arif et al., 1999). The authors speculated that high levels of HSP-specific antibodies may be vital in maintaining low levels of circulating BFT following the initial weeks of the acute infection in 36°C mice. The study's findings suggest that the antibody response to *T. cruzi* HSPs is not playing a critically protective role early in infection in 36°C mice, and that cytotoxic T lymphocytes specific for *T. cruzi* HSPs or HSP-associated peptides may be responsible for the control of the intracellular stages of the parasite in the first few weeks of infection (Arif et al., 1999).

A study conducted in 1998 by Powell showed that the adoptive transfer of whole spleen cells from mice that survived an otherwise lethal *T. cruzi* infection at 36°C conferred protection to naïve recipients. The adoptive transfer of whole spleen cells to lethally-infected naïve recipients maintained at room temperature imparted immunity that allowed for very low parasitemias, quick clearing of BFTs from the bloodstream, and a

100% survival rate. However, passive transfer of serum from surviving mice failed to confer any form of protection, a situation which suggests cell-mediated immune responses rather than humoral or antibody-mediated responses are responsible for the enhanced protection.

Numerous studies have shown that nitric oxide production by IFN-γ-stimulated macrophages (an important aspect of cell-mediated immunity) is vital in the control of intracellular stages of *T. cruzi*. This immunological response can be down-regulated by the inhibitory activity of the T_h2 cytokine IL-10. In the present study IFN-γ, IL-10, and nitric oxide levels were quantified to determine if this branch of cell-mediated immunity plays an important role in the protection seen in *T. cruzi*-infected mice maintained at elevated temperature.

Materials and Methods

Mice

Ten female C3HeB/FeL mice (Jackson Laboratories, Bar Harbor, Maine) were used in this study. The mice were divided into two groups (5 mice/group). One group of mice was maintained at room temperature (21°C), while the other group of mice was maintained in an environmental chamber held at 36°C. Both groups of mice were kept under a 12 hour light/12 hour dark photoperiodic cycle. Food and water were provided ad libitum. All mice were maintained and used in accordance with Public Health Service and local IACUC guidelines.

Parasitemias

Each mouse was infected on January 28, 2000 with 10^4 blood-form trypomastigotes of a Brazil strain of *T. cruzi* by intraperitoneal injection. Parasitemia levels were monitored in both groups of mice beginning on day 26 post infection and continuing every 3 to 6 days throughout the course of infection. A 4µl sample of blood was collected from the tail of each mouse for a direct parasite count. The sample was diluted with 96µl of Dulbecco's phosphate buffered saline (DPBS) (Sigma Chemical

Company, St. Louis, MO), and the number of parasites was counted using a hemacytometer.

Spleen Cell Preparation

On day 36 of infection all mice were anesthetized and approximately 1 ml of blood was drawn by cardiac puncture. Spleens were removed aseptically and placed into individual sterile petri dishes containing serum-free RPMI-1640 medium. The splenic capsule was disrupted using large forceps, releasing the cellular contents of the spleen into the medium. Spleen cells were collected into a 5-cc syringe through a 22-gauge needle and expelled through a 26-gauge needle into a sterile tube to obtain a single cell suspension of splenocytes. The cell suspension was centrifuged at 1500XG for 10 minutes. The supernatant was decanted and the pellet was resuspended in 0.5ml 1 X DPBS. Erythrocytes were lysed due to hypotonic shock by quickly adding 4.5 ml of distilled water. Tonicity was restored by adding 0.5 ml 10 X DPBS in less than 10 seconds. The suspension was centrifuged again at 1500XG for 10 minutes. Erythrocyte "ghosts" were removed mechanically. Remaining spleen cells were resuspended in 5.0 ml of complete RPMI-1640 (RPMI-1640 + 10% newborn calf serum + penicillin + streptomycin). A 20μl sample of the cell suspension was diluted in 380 μl of serum free RPMI-1640 and quantified using a hemacytometer. A single cell suspension was created by diluting the existing suspension to a final concentration of 6×10^6 SC/ml. Spleen cells were incubated (37°C and 5% CO_2) with or without the T cell mitogen concavalin A (Con A) for 60 hours.

Nitric Oxide Measurement

The production of nitric oxide was measured as the concentration of nitrite, the stable end product of NO synthase generated by reactive oxygen intermediates (ROIs). Spleen cells were adjusted to 6 x 10^6 SC/ml in RPMI-1640. A 100µl aliquot of spleen cells from each mouse was added to six wells of a sterile 96 well plate. A 100µl aliquot of Con A in complete RPMI-1640 (10 µg/ml) was added to three of these wells. The other wells received 100µl of complete RPMI-1640. Determination of nitrite concentration in supernatants was performed after 60 hours using the Griess assay. A 100µl volume of culture supernatant was transferred into corresponding wells of a 96 well microtiter plate (Falcon, Bectin Dickinson Laboratories, Lincoln Park, NJ). A 100µl volume of Griess reagent (40 mg/ml concentration) (Sigma) was added to each well, and care was taken to avoid contact with direct light. After a fifteen-minute incubation, the absorbance at 570 nm was determined by a microplate reader (BioRad, Hercules, CA). Nitrite concentration was determined using a standard curve of $NaNO_3$ (ranging in concentration from 2000 µg/ml to 62.5 µg/ml) prepared in complete RPMI.

Antigen-Capture ELISA for IFN-γ

Individual wells of a 96 well microtiter plate were incubated with 100µl of a 4 µg/ml concentration of rat anti-mouse IFN-γ, serving as the capture antibody, for approximately 24 hours at room temperature. After incubation, the plate was rinsed five times with 1 X DPBS. The plate was then blotted dry on a paper towel. A solution of 1% BSA, 5% sucrose in 1 X DPBS was used as the blocking solution. A 300 µl sample

of blocking solution was placed into each well and incubated for 2 hours at room temperature. After incubation, the plate was washed 5X as previously described.

A volume of 100μl of culture supernatant or serum samples was added in duplicate to rows A-F, while a standard curve of known concentrations of recombinant IFN-γ (2000 pg/ml to 62.5 pg/ml) was prepared in Row H (also in duplicate). After this addition, the plate was incubated for 2 hours at room temperature.

Biotinylated goat anti-mouse IFN-γ (400 ng/ml) diluted in 0.1% BSA, 0.05% Tween 20 in Tris-buffered saline with a pH of 7.3 served as the detection antibody. A 100μl volume of the detection antibody was added to each well, and the plate was incubated for another 2 hours at room temperature. The plate was washed again as described above.

A 100μl sample of streptavidin conjugated to horse radish peroxidase (1:2000) was added to each well, and the plate was incubated at room temperature for 20 minutes covered in aluminum foil to avoid direct contact with light. After washing the plate, 100μl of the substrate, Tetramethylbenzidine/peroxidase (Sigma), was added to each well. The plate was incubated for another 20-minute period under the same conditions as before. Absorbance values were determined by a microplate reader (BioRad) at a wavelength of 570 nm. Quantification of IFN-γ in the supernatants and sera was calculated by comparison to a standard curve of known concentrations of recombinant murine IFN-γ.

Antigen-Capture ELISA for IL-10

Individual wells of a 96-well microtiter plate were incubated with 100µl of a 4 µg/ml concentration of anti-mouse IL-10, serving as the capture antibody, for approximately 24 hours at 2-4°C. After incubation, the plate was rinsed five times with 1 X DPBS. The plate was then blotted dry on a paper towel. A solution of DPBS containing 1.0% BSA and .25% Tween 20 was used as the blocking solution. A 300 µl volume of blocking solution was placed into each well and incubated for 2 hours at 37°C. After incubation, the plate was washed 5X as previously described.

Culture supernatant or serum samples (100µl) were added in duplicate in rows A-F, while a standard curve of known concentrations of recombinant IL-10 (1600 pg/ml to 100 pg/ml) was prepared in Row H (also in duplicate). After this addition, the plate was incubated for 2 hours at 37°C.

Biotinylated goat anti-mouse IL-10 (0.2 µg/ml) diluted in blocking solution and 25 % heat-inactivated bovine calf serum served as the detection antibody. Detection antibody (100µl) was added to each well, and the plate was incubated for another 2 hours at 37°C. The plate was washed again as described above.

A 100µl volume of streptavidin conjugated to horse radish peroxidase (1:2000) was added to each well, and the plate was incubated at 37°C for 20 minutes. After washing the plate, 100µl of the substrate, Tetramethylbenzidine/peroxidase (Sigma), was added to each well. The plate was incubated for another 20-minute period at room temperature. Absorbance values were determined by a microplate reader (BioRad) at a wavelength of 570 nm. Quantification of IL-10 in the supernatants and sera was

calculated by comparison to a standard curve using known concentrations of recombinant murine IL-10.

Statistical Analysis

All statistical analyses were done using Systat (Wilkinson, 1992). A Discriminant Function Analysis was performed to determine if significant differences existed among the four groups of murine splenocytes, considering all three variables (NO production, IFN-γ synthesis and IL-10 synthesis). Predicted group membership was estimated a posteriori for all samples in splenocyte groups based on their squared Mahalanobis distance from the centroid of the source group. In addition, multivariate and univariate analyses were performed for all variables. Differences were considered significant at $P < 0.05$.

Results

Effect of Elevated Environmental Temperature on Parasitemia Levels

C3H mice infected with 10^4 blood-form trypomastigotes (BFTs) of a Brazil strain of *Trypanosoma cruzi* were placed at either 21°C (room temperature) or 36°C in order to further investigate the beneficial effects conferred to mice maintained at an elevated temperature. All mice were anesthetized on day 36 post infection (PI), and BFTs were quantified using a hemacytometer. Mice maintained at room temperature had mean peak parasitemia levels ranging from 18.75 X 10^4 BFT/ml to 68.75 X 10^4 BFT/ml (see Table 1). All mice maintained at 36°C showed no evidence of BFTs circulating in the blood. These mice also showed no obvious clinical symptoms of *T. cruzi* infection.

Room Temperature Mice	**36°C Mice**
Mouse 1: 68.75 x 10^4 BFT/ml	Mouse 1: 0 x 10^4 BFT/ml
Mouse 2: 43.75 x 10^4 BFT/ml	Mouse 2: 0 x 10^4 BFT/ml
Mouse 3: 18.75 x 10^4 BFT/ml	Mouse 3: 0 x 10^4 BFT/ml
Mouse 4: 18.75 x 10^4 BFT/ml	Mouse 4: 0 x 10^4 BFT/ml
Mouse 5: 68.75 x 10^4 BFT/ml	Mouse 5: 0 x 10^4 BFT/ml
SD = ± 54.93 x 10^4	SD = ± 0

Table 1. Effect of Elevated Environmental Temperature on Parasitemia. Parasitemia values were determined on day 36 post infection.

Nitric Oxide Production

The Griess assay was performed to determine the levels of nitrite (the stable end product of NO synthase generated by reactive oxygen intermediates) in murine splenocytes. As expected, murine splenocytes from both groups of mice (RT and 36°C) that were not stimulated with Con A showed similar low levels of nitrite in culture supernatant samples (see Figure 3). Con A-stimulated splenocytes from mice maintained at room temperature produced the highest levels (> 3000 μg/ml) of nitrite. In contrast, Con A-stimulated splenocytes from mice maintained at 36°C produced an average of approximately 2254 μg/ml of nitrite. Differences in NO production between these two groups of stimulated splenocytes were found to be statistically significant at a P value =< .001.

IFN-γ and IL-10 Synthesis

Synthesis of IFN-γ and IL-10 by cultured splenocytes was determined by an antigen-capture ELISA. An absence of IFN-γ synthesis was observed in non-stimulated murine splenocytes from both groups of mice (see Figure 4). Stimulated splenocytes from mice maintained at room temperature showed high levels of IFN-γ (> 5000 pg/ml), whereas stimulated splenocytes from mice maintained at 36°C exhibited approximately 3250 pg/ml of the cytokine. Differences in INF-γ levels were not statistically significant. As shown in Figure 5, higher levels of nitric oxide production tended to be correlated with higher levels of IFN-γ.

Average IL-10 levels were similar in stimulated splenocytes from both groups of mice (RT mice – 1213 pg/ml and 36°C mice – 1264 pg/ml) (see Figure 6). No IL-10

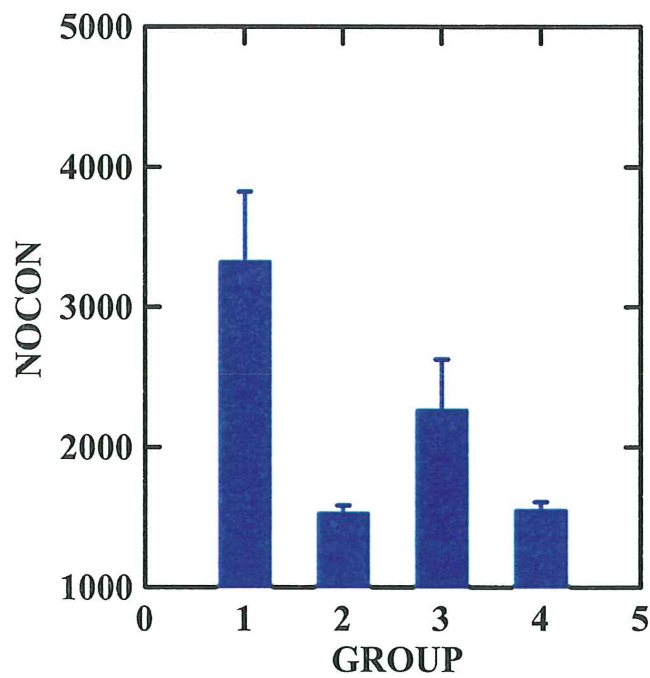

Figure 3. Nitric oxide production by murine splenocytes. Group 1- Con A-stimulated splenocytes from RT mice, Group 2- Non-stimulated splenocytes from RT mice, Group 3- Con A-stimulated splenocytes from 36°C mice, and Group 4- Non-stimulated splenocytes from 36°C. Nitric oxide production is given in μg/ml. Bars represent 2 X SE.

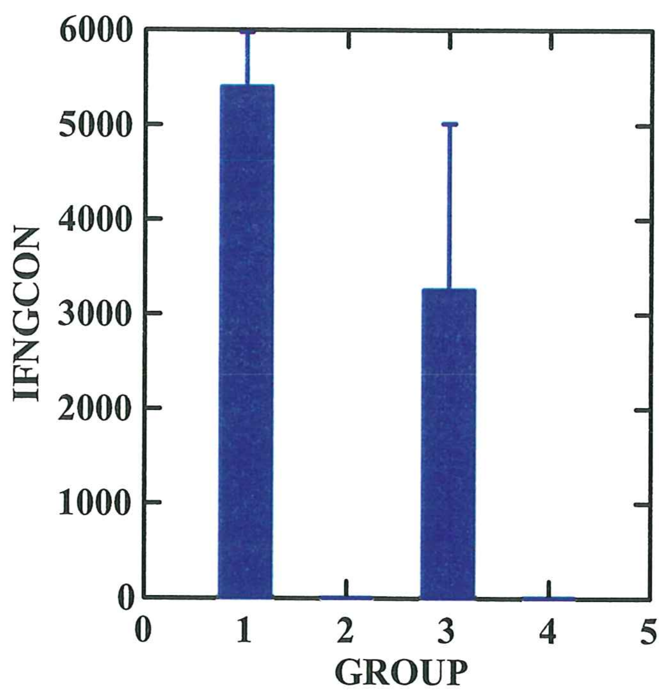

Figure 4. IFN-γ synthesis by murine splenocytes. Group 1- Con A-stimulated splenocytes from RT mice, Group 2- Non-stimulated splenocytes from RT mice, Group 3- Con A-stimulated splenocytes from 36°C mice, and Group 4- Non-stimulated splenocytes from 36°C. IFN-γ synthesis is given in pg/ml. Bars represent 2 X SE.

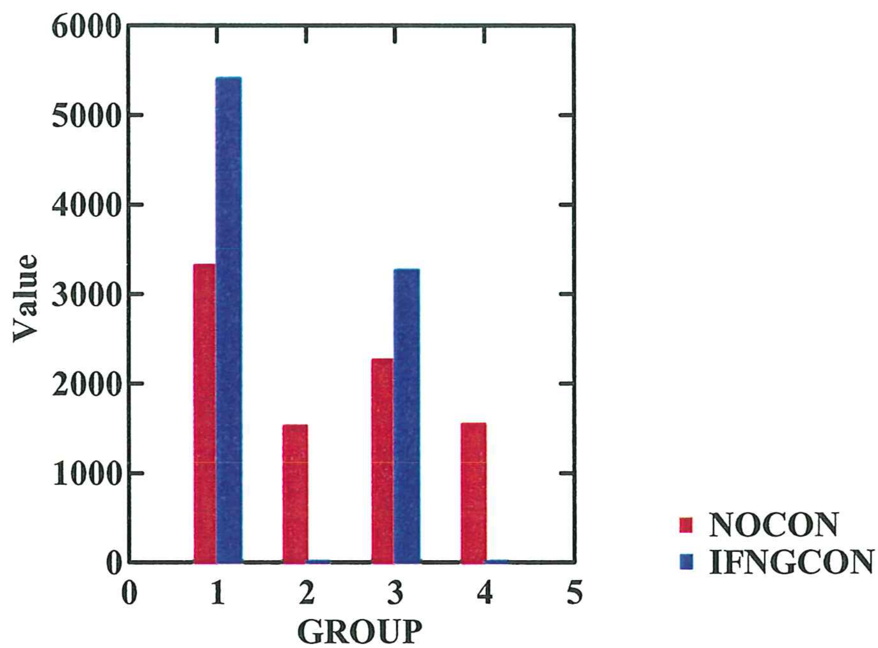

Figure 5. Comparison of NO and IFN-γ levels. Group 1- Con A-stimulated splenocytes from RT mice, Group 2- Non-stimulated splenocytes from RT mice, Group 3- Con A-stimulated splenocytes from 36°C mice, and Group 4- Non-stimulated splenocytes from 36°C. NO levels are given in μg/ml and IFN-γ levels are given in pg/ml.

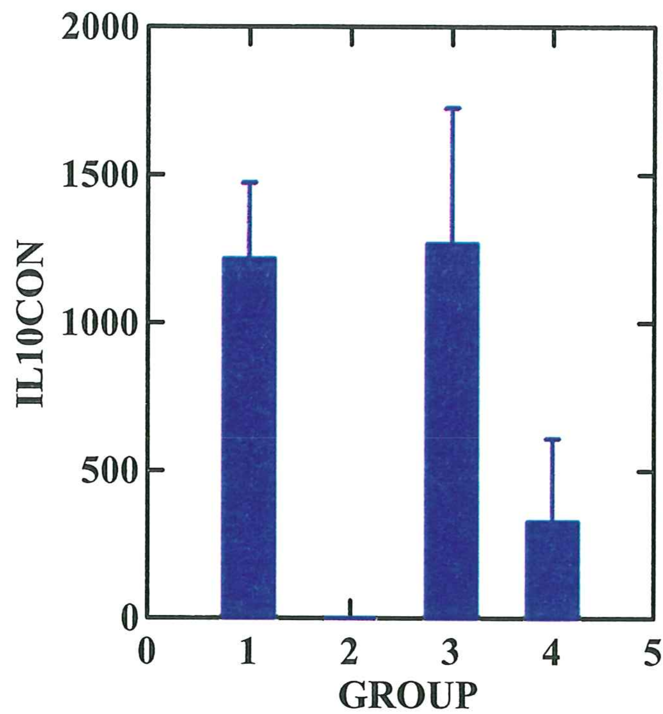

Figure 6. IL-10 synthesis by murine splenocytes. Group 1- Con A-stimulated splenocytes from RT mice, Group 2- Non-stimulated splenocytes from RT mice, Group 3- Con A-stimulated splenocytes from 36°C mice, and Group 4- Non-stimulated splenocytes from 36°C. IL-10 synthesis is given in pg/ml. Bars represent 2 X SE.

synthesis was observed in non-stimulated splenocytes from mice maintained at room temperature. However, a minimal amount of IL-10 synthesis was observed in non-stimulated splenocytes from mice maintained at 36°C with only two of the five mice producing recordable cytokine levels. In general, levels of IL-10 were approximately 4.5X and 2.5X lower than IFN-γ levels in RT and 36°C infected mice, respectively.

Statistical Analysis

The Discriminant Function Analysis showed that significant differences existed between murine splenocyte groups, with all variables (NO, IFN-γ and IL-10) contributing to the variance (see Table 2). As expected, no significant difference was detected in the non-stimulated splenocyte groups. Three samples (two from Group 3 and one from Group 4) were misclassified based on data obtained from their squared Mahalanobis distances. The multivariate analyses and analyses of individual variables also showed significant differences existed between groups (data not shown).

	Group 1	Group 2	Group 3	Group 4
Group 1	0.000			
Group 2	28.612**	0.000		
Group 3	11.143**	12.682**	0.000	
Group 4	26.170**	1.616	7.534*	0.000

Table 2. Discriminant Function Analysis.
* $P < .005$
** $P < .001$

Discussion

The results of this study provide additional evidence supporting enhanced protection conferred to C3HeB/FeJ (C3H) mice maintained at a constant environmental temperature of 36°C during the course of experimental Chagas' disease. Susceptible C3H mice infected with a Brazil strain of *Trypanosoma cruzi* experienced greatly reduced parasitemias as compared to infected mice maintained at room temperature. Room temperature (RT) mice developed parasitemias ranging from 18.75 x 10^4 BFT/ml to 68.75 x 10^4 BFT/ml, whereas all mice maintained at 36°C showed no evidence of blood-form trypomastigotes (BFTs) circulating in the blood and no obvious clinical symptoms of *T. cruzi* infection.

A number of studies have shown that highly susceptible C3H mice infected with a lethal Brazil strain of *T. cruzi* experience a delayed onset of parasitemias, decreased parasitemia levels, dramatically reduced numbers of BFTs, and increased longevity when maintained at an elevated environmental temperature of 36°C (Anderson and Kuhn, 1989; Dimock et al., 1991; Guo, 1994; Ming, 1994; Gao, 1995; Powell, 1998). Studies by Arif et al. (1999) also have shown that the beneficial immunological effects conferred to mice maintained at 36°C are not lost when the mice are returned to room temperature.

Their studies also demonstrated that the maintenance of mice at 36°C greatly reduces cardiac and skeletal muscle tissue pathology typically associated with acute *T. cruzi* infection. Maintenance of C3H mice at 36°C also has been shown to increase both parasite specific and non-specific immune responses (Anderson and Kuhn, 1989; Gao, 1995).

Previous studies have described nitric oxide (NO) production as crucial in the control of acute experimental Chagas' disease, and the primary effector mechanism involved in parasite killing in the macrophage (Plasman et al., 1994; Vespa et al., 1994; Petray et al., 1994; reviewed in James, 1995). However, the results of the present study clearly show that the primary mode of protection conferred to 36°C mice is not the enhancement of NO production. Stimulated splenocytes from RT mice showed an average NO level of 3311 μg/ml. In contrast, stimulated splenocytes from 36°C mice have an average NO level of only 2254 μg/ml.

A number of reports have determined that IFN-γ is a primary resistance factor in *T. cruzi* infections. The administration of recombinant IFN-γ increases the mice's resistance to the parasite, while neutralization of IFN-γ with monoclonal antibodies increases their susceptibility and shortens their survival time. These studies demonstrate that the cytokine is crucial in controlling the ongoing acute stage of infection (reviewed in Hoft et al., 2000). In the present study, antigen-capture ELISAs were performed to determine levels of IFN-γ and IL-10. The results of this study showed that stimulated splenocytes from RT mice - - mice which exhibited high parasitemia levels - - possess average IFN-γ levels of 5387 pg/ml. However, stimulated splenocytes from 36°C mice- - mice which exhibited total BFT clearing- - have average IFN-γ levels of 3250 pg/ml.

Results also indicated a correlation between higher levels of NO production and higher levels of IFN-γ. IL-10 levels obtained from stimulated splenocytes from both groups of mice exhibited similar levels with averages of 1213 pg/ml in RT mice and 1264 pg/ml in 36°C mice.

The present results suggested that enhanced levels of NO and IFN-γ are correlated with higher parasitemias and could actually have a deleterious effect on mice maintained at room temperature. Instead of increasing immunity and resistance to *T. cruzi*, host cell pathology seems to be implicated. A study conducted in 1997 by Hunter et al. showed that IL-10 knockout mice succumbed to infection earlier than wild type mice because of the overproduction of IFN-γ and IL-12. Further experimentation will be required to determine the role of NO and IFN-γ in *T. cruzi* infected mice maintained at 36°C.

Increasing evidence suggests that cell-mediated immune responses, especially cytotoxic T lymphocytes (CTL), may be responsible for the enhanced immunity seen in this model of experimental Chagas' disease. The *in vivo* depletion of T helper cells in mice maintained at 36°C resulted in 100% mortality (Guo, 1994), and the depletion of CD8+ T cells similarly reversed the positive effects associated with the elevated temperature (Ming, 1994). A study by Powell (1998) showed that adoptive transfer of whole spleen cells conferred protection to naïve recipients, while passive transfer of sera failed to confer protection. The study's results clearly demonstrated that cell-mediated immune responses rather than humoral or antibody-mediated responses are responsible for the enhanced protection.

Recent studies by Hoft et al. indicate that CD4+ T cells (T_h1 T helper cells) play a critical role in primary but not memory responses to *T. cruzi*. The authors suggest a role

for T_h1 cells in the optimal induction of CTL responses and cite previous studies implicating CD4+ T lymphocytes as providing important helper functions for the generation of effective CTLs. Additional studies have documented the importance of class I MHC-restricted CD8+ T cell responses in the protection against intracellular forms of the parasite. Studies also have shown that proteins released by the parasite in infected cells can serve as peptides for class I MHC antigen presentation, and can generate *T. cruzi*-specific CTLs (reviewed in Arif et al., 1999). Further experimentation is needed to determine the contribution of CD8+ T cells in *T. cruzi*-infected mice maintained at 36°C.

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