12-1-2001

The Effect of Lead Acetate on the Susceptibility of C3HeB/FeJ Mice Infected with the Parasite Trypanosoma Cruzi

Savonna Warren
Western Kentucky University

Follow this and additional works at: http://digitalcommons.wku.edu/theses

Part of the Medical Sciences Commons

Recommended Citation
http://digitalcommons.wku.edu/theses/628

This Thesis is brought to you for free and open access by TopSCHOLAR®. It has been accepted for inclusion in Masters Theses & Specialist Projects by an authorized administrator of TopSCHOLAR®. For more information, please contact topscholar@wku.edu.
THE EFFECT OF LEAD ACETATE ON THE SUSCEPTIBILITY OF C3HeB/FeJ MICE INFECTED WITH THE PARASITE, TRYPANOSOMA CRUZI

A Thesis

Presented to the Faculty of the Department of Biology

Western Kentucky University

Bowling Green, Kentucky

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Savonna L. Warren

December 2001
THE EFFECT OF LEAD ACETATE ON THE SUSCEPTIBILITY OF C3HeB/FeJ MICE INFECTED WITH THE PARASITE, *TRYPANOSOMA CRUZI*

Date Recommended  **Nov 20, 2001**

Cheryl D. Barse
Director of Thesis

**Elmer Greg**
Director of Graduate Studies  12/18/01
ACKNOWLEDGEMENTS

I would like to express my sincere thanks to my graduate committee in the Department of Biology at Western Kentucky University:

Dr. Cheryl Davis, my major advisor, for the gentle nudges that have kept me going this past year. Thank you so much for each and every word of support; without you I could not have completed this goal. Thanks for answering a seemingly inexhaustible number of questions and for never letting me give up. Thanks to Dr. Doug McElroy for his help with the statistical analysis of my data. Thank you for the support during my oral exam and thesis defense. Thanks go to Dr. Larry Elliot for all of the wonderful courses taught and for being so supportive during my undergraduate career.

I also would like to thank my friends and family members for the assistance during the past year. Thanks to my mom, Sandra, and my grandmother, Irene, for every inquiry and subsequent pep talk. Thanks to Melissa Clark at Princeton University for helping me through the maze of statistics. Thanks to the guys on "A" shift for keeping things running smoothly at work while I typed. A special thanks goes to Holly Clark for every word of encouragement and for being my greatest cheerleader. Thank you so much for being a part of this, the completion of my thesis.
# TABLE OF CONTENTS

## INTRODUCTION

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chagas' Disease</td>
<td>1</td>
</tr>
<tr>
<td>Development</td>
<td>2</td>
</tr>
<tr>
<td>Transmission</td>
<td>3</td>
</tr>
<tr>
<td>Trypanosoma cruzi</td>
<td>6</td>
</tr>
<tr>
<td>Immune Response</td>
<td>8</td>
</tr>
<tr>
<td>Antibodies</td>
<td>10</td>
</tr>
<tr>
<td>Role of Cytotoxic T cells</td>
<td>11</td>
</tr>
<tr>
<td>Cytokine Regulation</td>
<td>12</td>
</tr>
<tr>
<td>Lead</td>
<td>13</td>
</tr>
<tr>
<td>Epidemiology</td>
<td>13</td>
</tr>
<tr>
<td>Toxicity</td>
<td>14</td>
</tr>
<tr>
<td>Effect on Immune Response</td>
<td>15</td>
</tr>
</tbody>
</table>

## MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>18</td>
</tr>
<tr>
<td>Data collection</td>
<td>18</td>
</tr>
<tr>
<td>Water consumption</td>
<td>19</td>
</tr>
<tr>
<td>Survival</td>
<td>19</td>
</tr>
<tr>
<td>Weight</td>
<td>19</td>
</tr>
</tbody>
</table>
RESULTS

Water consumption .................................................. 25
Survival ...................................................................... 25
Weight ........................................................................ 25
Parasitemia ................................................................. 26
Antibody ................................................................. 27

DISCUSSION .................................................................. 33

LITERATURE CITED ...................................................... 37
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURES</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Average volume of drinking water consumed per group</td>
<td>28</td>
</tr>
<tr>
<td>2. Impact of lead acetate on survival</td>
<td>29</td>
</tr>
<tr>
<td>3. Impact of lead acetate on weight</td>
<td>30</td>
</tr>
<tr>
<td>4. Impact of lead acetate on number of circulating parasites</td>
<td>31</td>
</tr>
<tr>
<td>5. Impact of lead acetate on antibody production in response to infection</td>
<td>32</td>
</tr>
</tbody>
</table>
Lead has been mined by man for centuries with evidence of its use dating back 6000 years. Chronic exposure to lead can result in encephalopathy, anemia, and nephropathy. Today, cases of lead poisoning are uncommon; however, lead continues to be incorporated into the body through subclinical exposures. These low dosages of lead have been shown to have a deleterious effect on the immune system. Experimental animals exposed to low levels of lead are unable to effectively respond to a number of bacterial and viral challenges. The present study focuses on the relationship between lead and parasitic infection. Parasitemia, mortality, serum antibody levels, and host growth rate were examined using 25 C3HeB/FeJ mice orally administered lead acetate and subsequently infected with Trypanosoma cruzi. Mice were divided into groups of five and given varying concentrations of lead acetate (0 p.p.m., 0.01 p.p.m., 0.1 p.p.m., 1 p.p.m. and 10 p.p.m.) for 30 days whereupon they were infected with $1 \times 10^4$ BFTs of the Brazil strain of T. cruzi. Statistical analysis determined that the low dosages of lead did not negatively impact the host's immune system, thereby resulting in increased
infection and death. However, further research is needed to determine the effect that higher lead dosage may have on host resistance to disease.
INTRODUCTION

Chagas' Disease

In 1908 Oswaldo Cruz, founder of the Manguinhos Institute, commissioned Carlos Chagas to work as a malaria control officer in the interior of Minas Gerais State. While there, Chagas discovered the presence of large, voracious, blood-feeding insects infesting the houses of the poorer class. Recent discoveries had linked arthropods as vectors of human disease, leading Chagas to dissect and examine these insects. Chagas found numerous flagellates in the feces and immediately sent infected bugs to the laboratories of the Manguinhos Institute in Rio (Miles, 1983). These parasite-infected insects were fed to primates free from infection. After several weeks, the same flagellate was recovered from the blood stream of the primates. All of the primates died two to three months after infection. Following these results, Chagas examined the blood of humans and domestic animals living in the insect-infested dwellings. Chagas found the parasite in the blood stream of a two-year old girl, Bernice, who had originally been diagnosed with acute malaria (Minchin, 1910). Chagas reported his findings to the Manguinhos Institute and named the parasite Trypanosoma cruzi in honor of Oswaldo Cruz. The disease caused by the parasite became known as Chagas' disease (Miles, 1983).
Members of the National Academy of Medicine disputed Chagas’ claims. In 1916 a prominent German microbiologist, Krause, denied the existence of Chagas’ disease after finding no cases of the disease. Chagas went on to replace Oswaldo Cruz at the Manguinhos Institute and began the difficult task of controlling Spanish Fever in Rio de Janeiro. Chagas’ disease was forgotten for almost twenty years. Between 1931 and 1936, Johnson and Rivas described more than a thousand cases of Chagas’ disease leading to its rediscovery (Wendel, 1992).

Development

Chagas’ disease is a parasitic infection caused by the protistan Trypanosoma cruzi and is transmitted to vertebrate hosts by hematophagus insects of the family Reduviidae. It is estimated that 24 million people are affected by Chagas’ disease with cases documented in South and Central America as well as some parts of Mexico (Ouaissi et al., 1991). The disease is usually contracted when the feces of infected insects comes in contact with either the mucosa or skin. The fecal material contains the infectious form of the parasite and is deposited on the skin of the victim following a blood meal. Penetration by the parasite occurs when the feces is rubbed into the puncture wound produced by the insect bite or into mucous membranes (Wendel, 1992). At the site of entry a cutaneous chagoma characterized by a small, reddish, slight painful nodule may occur. The chagoma is often accompanied by swelling of the satellite lymph nodes (Schmunis, 1991). If the parasite invades via the mucous membrane of the eye unilateral conjunctivitis develops. The swollen, red area encircling the eye is a classic symptom of Chagas’ disease and is known as Romana’s sign (Miles, 1983).
Acute Chagas’ disease follows a short incubation period of seven to nine days and varies from asymptomatic to fatal. When present, symptoms may include fever, lymphadenopathy, blood parasitemia, and, depending on the route of entry, a cutaneous chagoma or Romana’s sign. Also, enlargement of the liver and spleen and acute myocarditis or meningoencephalitis may occur. Mortality is usually low, around 10%, except for young children with cardiac or central nervous system involvement (Schmunis, 1991). The indeterminate phase has little evidence of infection and may last for life. Chagas’ first patient, Bernice, lived until over the age of 70 and exhibited none of the detectable abnormalities attributed to Chagas’ disease. After a latent period of years or decades, up to 40% of people infected with Trypanosoma cruzi will develop chronic Chagas’ disease (Miles, 1983). Myocardial neural plexes and the digestive system plexes including the esophagus and the colon are most often colonized by the parasite (Bastien, 1998). This colonization leads to the development of the megasyndromes seen in chronic Chagas’ patients: megaesophagus, megacardia, or megacolon. Those patients developing megaesophagus or megacolon have a greater chance of survival. These symptoms are easily recognized, and the affected tissue can be surgically removed. Megacardia, however, is not usually detected, and the patient dies from heart failure when the walls of the heart become so thin that the heart bursts from simple ventricular pressure (Miles, 1983).

**Transmission**

There are two main routes of transmission for American Trypanosomiasis. The most common route is exposure to the feces of an infected triatomine insect. Triatomines
are insects of the order Hemiptera, family Reduviidae and subfamily Triatominae. Over ninety million people are at risk of contracting Chagas' disease due to the wide range of these insects (Wendel, 1992). Infected triatomines can be found from latitude 42 N to latitude 43 S (Schmunis, 1991). However, of the 118 species of triatomines only a small number are epidemiologically significant as vectors of human Chagas' disease (Wendel, 1992). The most important vectors are *Triatoma infestans*, *T. sordida*, *T. dimidiata*, *Panstrongylus megistus*, *Thodnius pallescens*, and *Rhodnius prolixus* (Schmunis, 1991). These triatomines readily infest houses of the poorer class. *Rhodnius prolixus* can be found in palm roofs of houses while *P. megistus* frequents timber-framed, mud walls. *Triatoma infestans* will colonize relatively good quality houses with plastered walls and tiled roofs. Infestations of houses will include several thousand triatomines. Although the insects may not be easily seen they are evidenced by the production of a 'fecal rain.' The colonies literally produce a rain of infected feces upon the unfortunate inhabitants (Miles, 1983).

The socioeconomic difficulties in these rural areas give rise to a second route of transmission. In an effort to escape poverty, illiteracy, and disease, infected individuals migrate to urban regions. Immigration to the United States is also a survival strategy for many individuals from Latin American countries. There are an estimated 100,000 cases of chronic Chagas' disease in the US at this time. Migrants from rural areas make up a large percentage of the unskilled labor force and are likely to be underemployed. These individuals donate infected blood for money leading to the transmission of disease through contaminated blood. In Latin America, transmission via infected blood has become a huge problem. In Argentina, Brazil, and Chile 14 to 18% of persons receiving
blood transfusions will become infected with *T. cruzi*. The number of individuals becoming infected is 49% in Bolivia where 62% of the blood donors test positive for *T. cruzi* (Schmunis, 1991). As the number of infected Latin American immigrants continues to increase in the United States, the incidence of transmission-acquired Chagas' disease would be expected to increase. A slight reduction in the occurrence of Chagas' disease has occurred in recent years due to vector control and bloodbank screenings. However, obstacles such as behavioral differences among vector species, operational costs of maintaining active control programs, and the existence of multiple animal reservoirs complicate the eradication of the disease (Wizel, 1998).

Another parameter, which could affect transmission of disease, is global warming. The increased burning of fossil fuels has caused a rise in carbon dioxide, methane, hydrocarbons, and oxides of nitrogen. This increase of heat-trapping greenhouse gases has resulted in an increase in global temperature of 1° C. The cooling trend of the last 1000 years has reversed (Epstein, 2001).

Cases of disease involving two or more species will increase due to global warming. Vector-borne pathogens especially will be affected as a portion of the life cycle is spent in an invertebrate host, whose body temperature will closely reflect that of ambient temperature. An increase in global temperature will result in an increase in transmission seasons, as insects will survive a longer period of time. Also an increase in vector range will occur as areas once too cool to sustain the vector increase in temperature (Patz and Reisen, 2001). Warmer temperatures also will result in increased insect reproduction and biting activity leading to an increase in pathogen exposure (Epstein, 2001).
Trypanosoma cruzi

Trypanosoma cruzi is characterized by the presence of one flagellum, a singular organelle, the kinetoplast, and one nucleus. The flagellum emerges from the flagellar pocket, a special invagination involved in ingestion of nutrients. Pinocytotic vesicles are present in this region and are involved in the incorporation of macromolecules from the external medium. The kinetoplast is a fibrous network and comprises approximately 20-25% of the parasite's total DNA. The kinetoplast DNA (K-DNA) molecule is organized into minicircles and maxicircles. Evidence suggests that maxicircles code for enzymes necessary for metabolism. However, the role of the minicircle has not yet been established. Research shows that parasitic forms lacking a kinetoplast are unable to complete a normal cycle, demonstrating that K-DNA is essential to the viability of the parasite (Wendel, 1992).

During its life cycle, Trypanosoma cruzi exists in one of three forms. The trypomastigote is the infectious form of the parasite. It is 20 microns long and 3 microns wide with a free flagellum at the end of the body attached to an undulating membrane. The nucleus and the kinetoplast are located at the posterior end of the parasite. The trypomastigote has a basket-shaped kinetoplast due to the arrangement of the DNA loops. The flagellum allows the parasite to attach to the insect's intestinal wall and pass from the hindgut into the feces. This form travels through the infected person's bloodstream and then colonizes muscle and neuronal tissues. Once inside the host cell, the metacyclic trypomastigote transforms to the amastigote. Amastigotes are intracellular, round in shape, do not possess a flagellum, and are approximately 1.5 to 5 microns long (Bastien,
The amastigotes replicate by binary fission in muscle tissue and transform back to the trypomastigote stage prior to erupting from the cell and entering the bloodstream where they are able to move on to other tissue cells (Souto-Padron et al., 1989). This process provides for rapid proliferation. In the infected host there are alternating periods of parasitic division in tissues and the presence of numerous flagellates in the bloodstream (Minchin, 1910). Triatomines ingest circulating trypomastigotes from infected individuals while taking a blood meal, becoming infected for life. The trypomastigotes travel to the insect’s midgut and transform into epimastigotes. The epimastigote form is capable of surviving in the insect’s intestines. The flagellum of the epimastigote is attached near the center of the body. Epimastigotes multiply by binary fission and have a central nucleus and kinetoplast (Bastien, 1998). The kinetoplast is rod-shaped in both the amastigote and the epimastigotes stages (Wendel, 1992).

Epimastigotes are 10 to 20 microns long and grow an additional 10 microns as they travel from the insect’s midgut to the hindgut. In the hindgut, epimastigotes transform into the metacyclic trypomastigote stage, completing the life cycle of the trypanosome. The insect stage of *T. cruzi* lasts approximately 16 days and does no apparent harm to the insect (Bastien, 1998).

*Trypanosoma cruzi* exists as a pool of strains rather than as a homogeneous population. Strains of *T. cruzi* have distinct characteristics that play a role in the morphology of blood forms, curves of parasitemias, virulence, pathogenicity and sensitivity to chemotherapeutic drugs (Wendel, 1992). These differences in strains may explain why there are many cases of megasyndrome in chronic chagistic patients in Brazil, whereas there are no cases of megasyndrome in Venezuela (Miles, 1983).
Immune Response

As there is no cure for Chagas' disease, much research has been centered on uncovering the relationship between *Trypanosoma cruzi* and the body's immune system. Some investigators have demonstrated that the body can produce a partial immunity to *T. cruzi*, and in many cases the severe pathologies of Chagas' disease are avoided (Bastien, 1998). Partial immunity can be achieved through activation of T and B lymphocytes, activation of macrophages, and production of cytokines. Passive immunity can be conferred to a host by injection of purified IgG or sera from an infected individual as evidenced by lower parasitemias and a reduction in mortality (Muller et al., 1986). Passively immunized individuals will develop Chagas' disease, but do survive longer than non-immunized controls. In endemic areas this passive immunity would be beneficial due to the fact that complete removal of the parasite would re-expose the individual to the lethal dangers of acute Chagas' disease (Bastien, 1998).

*Trypanosoma cruzi* has evolved several adaptive mechanisms to avoid removal from the infected host's body by the immune system. Evasion of the immune system, stimulation of an autoimmune response, and suppression of the immune system are all methods that have been proposed to be used by the parasite.

By incorporating certain host cell membrane proteins onto the surface of the parasite, *T. cruzi* can mask its antigenic signal. The parasite invades monocytes rendering it undetectable. Amastigotes then spread into other host tissues allowing for rapid proliferation of the parasite. During the early stages of infection, polyclonal proliferation of nonspecific antibodies has been shown (Hontebeyrie-Joskowicz, 1992).
Trypomastigotes are present in the blood stream for only a short time, giving the immune system little time to develop parasite-specific immunoglobulins. Lysis of trypomastigotes by the complement cascade system is antibody-dependent. Without specific antibodies to bind to the parasite, complement cannot destroy the trypomastigotes. *Trypanosoma cruzi* can then invade tissue and proliferate in the amastigote form (Bastiens, 1998). Research has shown that antibodies bind more readily to the amastigote and epimastigote forms indicating that the trypomastigote is able to evade the immune system (Israelski et al., 1988). Trypomastigotes produce soluble antigens that are released into the blood stream. The secreted antigens succeed in diverting the antibody response from the parasite itself (Cerban et al., 1992).

Autoimmunity results when the body’s powerful immune system turns on itself instead of riddling the body of foreign agents. It has been hypothesized that the destructive symptoms of Chagas’ disease are the results of autoimmunity (Bastein, 1998). Myocardial and neuronal tissues express antigens that are common with *T. cruzi* (Reis et al., 1993). During the acute phase of the disease, B and T cells produce antibodies that attack epitopes common to *T. cruzi* and the body (Bastien, 1998). However, recent studies by Tarleton and co-workers have indicated that pathogenesis in Chagas’ disease is not the result of autoimmunity. The main support of the autoimmune hypothesis is that signs of disease are evident in tissues not actively colonized by the parasite. However, recent immunohistochemistry, whole tissue, and in situ PCR analyses have demonstrated the presence of parasites at the site of disease. *Trypanosoma cruzi* DNA was found in heart tissue of seropositive cadavers that were diagnosed with chronic cardiomyopathy. No DNA was found in the cardial tissue of those patients that lacked evidence of Chagas’
cardiopathy. Also, *T. cruzi* DNA was present in the visceral tissues of cadavers diagnosed with megacolon or megaesophagus, but was not found in Chagas’ patients that died resulting from cardiopathy with no evidence of disease in the esophagus and colon. Furthermore, enhancing the anti-parasite response results in a decrease in severity of chronic disease. If the autoimmune hypothesis were true then an enhancement of the host's immune system should result in an increase in severity of disease (Tarleton, 2001).

Some studies have shown that during early development of the disease a profound and nonspecific suppression of the immune system occurs. One mechanism may be a suppressor macrophage with the capacity to down-regulate the response of normal lymphocytes, specifically helper T cells. After two weeks of initial infection with trypanosomes, spleen cells are unable to produce interleukin-2 (IL-2) a cytokine secreted by T cells. Following infection, mice challenged with sheep RBC's were unable to elicit an immune response. The addition of IL2 restores the ability of the mice to respond to antigenic challenge, including a 2 fold increase in the presence of anti- *Trypanosoma cruzi*-specific T cells (Kuhn, 1989).

**Antibodies**

Antibodies play an important role in the immune response to *T. cruzi* contributing to complement-mediated lytic activity, cell-cytotoxicity, and phagocytosis (Jorge et al., 1993). In response to most other protozoan infections, IgM is typically produced prior to IgG. However, research with *T. cruzi* has shown that IgM and IgG reach peak titers at the same time. IgM titers decreased rapidly and by day 90 of infection, sera was negative for IgM, indicating a more dominant role in parasitic clearance for IgG (Israelski et al.,
Antibody isotopes of IgG involved in the clearance of blood parasites include IgG1, IgG2a, IgG2b, IgG3 and IgG4. These immunoglobulins aid in lysing circulating trypomastigotes (Jorge et al., 1993). IgG1 appears to be produced in more abundance than the other subsets (Cerban et al., 1992).

Trypomastigotes present in susceptible hosts are normally resistant to the lytic effects of complement. However, during chronic infections, antibodies that render the trypomastigote sensitive to complement-mediated lysis are produced. Experiments have demonstrated that IgG binds to the surface of the parasite enabling complement from fresh human sera to lyse trypomastigotes. However, if immunoglobulin is not present on the surface of the parasite, lysis does not occur. The trypomastigote has C3b- and C4b-binding proteins located on its surface. When C3b is bound to the surface of the parasite it cannot interact with factor B thereby inhibiting formation of C3 convertase. Parasite-specific lytic antibody binds to the surface of the trypomastigote blocking the receptor sites for C3b and C4b. C3 convertase is formed and lysis of the parasite occurs as a result of the complement cascade system (Krautz et al., 2000).

**Role of Cytotoxic T cells**

The humoral immune response attempts to eliminate circulating trypomastigotes from the host's blood stream. The intracellular parasite form, the amastigote, is controlled by the cellular immune response, which destroys parasite-infected cells. Experimental models of Chagas' disease have established the necessity of CD8$^+$ T cells in controlling tissue parasitemia as cytotoxic T lymphocytes (CTL). Rottenberg in 1993, demonstrated that CD8$^+$ mice are susceptible to *T. cruzi* infection, displaying striking
increases in parasite growth and mortality. In 1998, Wizel et al. discovered two parasite-specific targets for CTL on the surface of the parasite's intracellular form, amastigote surface protein (ASP)-1 and ASP-2. *Trypanosoma cruzi* replication takes place in the cytoplasm in the amastigote form, making CTL important in the eradication of the parasite from the host. As the amastigote infests a number of cells that do not express surface class II MHC molecules, CTL are vital for the recognition of parasite peptides presented by class I MHC.

**Cytokine Regulation**

Cytokines are secreted proteins that mediate interactions among cells of the immune system. Several cytokines have been shown to have important roles in the host’s immune response to *T. cruzi* infection. Interleukin 2 (IL-2) deficiency has been linked to the nonspecific immunosuppression characteristic of Chagas’ disease. IL-2 is a potent T-cell growth factor and enhances the T helper cell and natural killer (NK) cell activity. Mice injected with IL-2 following infection of *T. cruzi* were shown to have significantly reduced parasitemia levels and increased longevity as compared to mice not administered IL-2 (Hulsebos et al., 1989). Interferon gamma (INF-γ) is secreted by *T. cruzi* resistant CDBL/6 mice, but not susceptible BALB/c mice (Hoft et al., 2000). In vitro administration of IFN-γ activates macrophages to kill or inhibit intracellular parasite growth. Trypanocidal activation of macrophages is reduced upon in vivo administration of anti-IFN-γ (Petray et al., 1993). Interleukin 10 (IL-10) blocks the production of IFN-γ decreasing the ability of macrophages to destroy intracellular *T. cruzi* (Reed et al., 1994).
Lead

Lead occurs naturally in the earth’s crust in small concentrations. However, it has been mined and disseminated throughout the environment collecting in structural tissues in plants, animals, and man. The extent of worldwide contamination of lead has increased dramatically in the last 60 years (Healy and Aslam, 1981). Approximately 4.5 million tons of refined lead are consumed annually. Urban air has now been found to contain 20,000 times more lead since the beginning of modern technology. Studies have shown that the lead contamination of the polar ice cap and remote oceanic waters has increased more than 400 fold. An average person from the United States has been found to have a total body lead burden 500-1000 times greater than pretechnological man (Boutron et al., 1991).

Epidemiology

Lead has been used commercially in paint, batteries, printing, plastics, putty, plaster, solder, and ceramic glazes (Koller, 1979). However, the greatest source of lead contamination is the use of tetraethyl lead or tetramethyl lead in gasoline as an antiknock agent. In the United States, catalytic converters were made mandatory on cars produced after 1975 in an effort to decrease this source of lead contamination. However, a single older, badly maintained automobile emits as much toxic gas as 40 newer, cleaner cars. Also, the number of vehicles on the road has increased since 1975, as well as the distance traveled (Russel-Jones, 1982). The inhalation of lead fallout from automobiles is a principle route of exposure. Children in particular are affected by airborne pollution because oxygen consumption per kg body weight in children is about twice that in adults.
In addition, the absorption rate of lead by children is about 30% higher than adults (Day et al., 1975).

Lead also can enter the body through ingestion of contaminated food or drinking water. Most lead pollution in food is due to lead fallout from gasoline emissions. Plants have natural barriers in the roots that prevent uptake of lead in soil. However, airborne lead bypasses this barrier and deposits directly on leaves. Consumption of contaminated food as well as meat from animals that have grazed on polluted grass can greatly increase lead in the body (Moore, 1979). Around 10% of dietary lead comes from contaminated drinking water. Many houses have old, lead pipes, which release lead into drinking water. Thus, the content of lead at the tap may be much higher than when the water leaves the pumping station (Bryce-Smith and Waldron, 1974). Ingestion of paint chips, putty, plaster, colored magazine pages, and lead-contaminated soil increase the risk of chronic lead poisoning in children (Koller, 1979). The percentage of ingested lead actually absorbed by children is up to 50%, whereas adults only absorb 5-10% (Gloag, 1980). However, more lead is retained than excreted leading to a progressive accumulation of lead (Bryce-Smith and Waldron, 1974).

Toxicity

Lead metabolism can closely mimic calcium metabolism, particularly at the receptor sites of membranes. Lead can replace calcium and adversely affect both neuromuscular and synaptic transmissions, thus explaining why lead progressively accumulates in bone tissue (Bryce-Smith and Waldron, 1974). Bone lead is gradually released into the surrounding soft tissue. Therefore, lead could be a contributing factor in
the development of motor neuron disease, bone fractures, and wasting bone disease (Cambell et al., 1970). Lead also acts as a potent neurotoxin, particularly prior to birth and during early development (Bryce-Smith, 1983). Chronic lead exposure has been linked to early development of pre-senile dementia. The adult brain also is vulnerable to the neurotoxic effects of lead exposure. Symptoms include restlessness, talkativeness, mania, over anxiety, aggression, suicidal tendencies and some forms of schizophrenia (Hunter, 1969).

**Effect on Immune Response**

Exposure to lead is known to inhibit the immune system, decreasing host resistance to a number of challenges. Inhalation of lead chloride has been shown to impair elimination of bacteria from the lung. Rodents exposed to lead demonstrated a decrease in resistance to bacterial and viral challenges. Rats experienced an increased susceptibility to *Escherichia coli*, while mice were shown to be less resistant to *Salmonella typhimurium*, encephalomyocardial virus, and *Hexamita muris*, a common protozoan parasite found in the intestines. Rabbits fed lead acetate in drinking water for 70 days exhibited marked immunosuppression when challenged with pseudorabies virus (Pelletier et al., 1990).

Lead causes a decrease in immunological memory and interferes with complement receptors on B lymphocytes in mice. Lead interferes with phagocytic activity of polymorphonuclear leukocytes and reduces lysozyme activity. Animals exposed to lead produce lower levels of serum globulin and complement (Koller, 1980). Oral administration of lead acetate decreases antibody titers, specifically IgG and IgM, at
low concentrations (Blakley et al., 1979). IgG has been shown to be affected more than IgM (Koller et al., 1976).

The effect of lead on the response of the immune system has been documented with regard to bacterial, viral, and tumoral challenges. However, little research has been performed analyzing lead's effect on the host's immune system during parasitic infection. In 1996, Ellis conducted a study on the effect of lead acetate on host susceptibility to *T. cruzi*. Lead acetate was administered orally to female resistant C57BL/6 mice for 3 weeks. One group received no lead acetate as a control. During the fourth week, all mice were infected with $1 \times 10^4$ BFT of the Brazil strain of *T. cruzi*. Parasitemias were started on day 14pi and continued on a 3 to 4 day interval. Parasite-specific antibody analysis was performed using ELISA. High numbers of blood form-trypomastigotes were seen in groups administered 100 and 1000 p.p.m. lead acetate. However, all mice experienced low mortality and no statistical difference in antibody concentration. The present study was designed to analyze the effect of oral administration of lead acetate on C3HeB/FeJ mice when infected with *T. cruzi*. The C3HeB/FeJ strain is very susceptible to the Brazil strain of *T. cruzi* with high parasitemias and mortality occurring by days 35-40 of infection. Several studies have indicated that IgM and IgG play an important role in the response to *T. cruzi*. However, *T. cruzi* trypomastigotes have an excellent defense mechanism against circulating antibodies. Studies also have shown that lead inhibits antibody proliferation at low concentrations, specifically IgM and IgG, which could result in exacerbation of experimental Chagas' disease. In order to determine if lead acetate would have a detrimental impact during murine infection with *T. cruzi*, parasitemia counts, serum antibody concentration, weight, and mortality rates were
analyzed. In addition, varying concentrations of lead acetate were administered to
determine if results were dosage-dependent.
MATERIALS AND METHODS

Mice

Twenty-five C3HeB/FeJ female mice were obtained at 5 weeks of age from Jackson Laboratory in Bar Harbor, Maine. All mice were acclimated on deionized water and laboratory rodent chow (PMI Feeds, St. Louis, Missouri) for two weeks. Mice were maintained in the animal control laboratory at a constant temperature of 23° +/- 1° C and a photoperiod of 12 hours light/12 hours dark. Following acclimation, mice were randomly distributed to one of the five test groups, each consisting of five mice.

A stock solution of 10 p.p.m. lead was prepared by dissolving 20 mg of lead acetate in 2 L of deionized water. The remaining test concentrations were diluted from the prepared solution. The lead solutions were administered to the mice as drinking water. Test groups and corresponding lead concentrations were as follows: Group A - 0 p.p.m., group B - 0.01 p.p.m., group C - 0.1 p.p.m., group D - 1 p.p.m., group E - 10 p.p.m.. Following lead exposure for thirty days, all mice received an intraperitoneal injection of $1 \times 10^4$ blood-form trypomastigotes (BFT's) of *Trypanosoma cruzi*, Brazil strain. The strain of *T. cruzi* used is maintained continuously in our laboratory as a stock infection in C3HeB/FeJ female mice.

Data collection
**Water consumption**

Each group of mice received an initial 200 ml of drinking water containing the corresponding concentration of lead. Water bottles were monitored and refilled approximately every 7 days and the volume of water consumed was recorded.

**Survival**

Number of surviving mice per group was recorded every 3 to 4 days. Percent survival was calculated.

**Weight**

Initial weight for each mouse was recorded on day 28 pre-infection (day 28 pre). Administration of lead solution as drinking water began day 28 pre after the initial weight was recorded. Weight data was collected for each mouse approximately every 7 days throughout the course of infection.

**Parasitemia**

Parasitemia counts were recorded every 3 to 4 days beginning day 14 pi. Blood from each mouse was extracted from the tail vein by clipping the end of the tail and collecting the blood with capillary tubes. Ninety-six μl of Dulbecco's Phosphate Buffered Saline (DPBS) was mixed with 4 μl of blood in Eppendorf tubes, creating a 1/25 dilution. Approximately 20 μl of cell suspension was placed on a hemacytometer (Fisher Scientific, Pittsburgh, Pennsylvania) and parasites in the four large corner squares were counted. The following equation was used to convert this raw number to number of
cells per ml of blood: cells/ml blood = (average number per large square) * 10^4/ml * 1/dilution.

**Antibody concentration**

Serum from each surviving mouse was collected on day 32 pi and analyzed for antibody concentration by ELISA. The procedures for the preparation of the *T. cruzi* antigen extract, preparation of the murine plasma, and the indirect ELISA follow.

**Preparation of *T. cruzi* antigen extract**

A PSC3H murine fibroblast cell line infected with *T. cruzi* trypomastigotes was cultured in RPMI-1640 medium (Sigma) supplemented with 10% newborn calf serum (Microbiological Associates, Walkersville, Maryland), 25 mM HEPES (Sigma), and penicillin/streptomycin. Culture supernatants containing trypomastigotes were collected and filtered. The filtered supernatant was centrifuged for 30 minutes at 1800 x g concentrating the parasites into a pellet. The pellet was resuspended in 1 ml of DPBS, placed in a 1.7 ml Eppendorf tube and centrifuged for 15 minutes at 2000 x g. The pellets were resuspended two more times, combined and resuspended in ice-cold DBPS containing 0.5% Triton X and 2mM phenylmethylsulfonyl fluoride (Sigma). The suspension was transferred to a 15 ml centrifuge tube and incubated for 30 minutes in an ice bath and vortexed periodically. The suspension created was spun at 8,000 rpm for 15 minutes to remove insoluble material and the supernatant was collected. A Bio-Rad protein determination assay was performed on the antigen extract using bovine serum
albumin as a standard. The antigen extract was diluted to a working concentration of 50 µg/ml in DPBS for use in an indirect enzyme linked immunosorbent assay (ELISA).

**Preparation of murine plasma**

Blood from each surviving mouse was collected from the tail vein on day 32 post infection for use in indirect ELISA. Four µl of blood was combined with 96µl of DPBS in 1.7 ml Eppendorf tubes and thoroughly mixed. The solution was centrifuged at 5,000 rpm for 10 minutes. Plasma samples were labeled and stored at −4°C until analyzed.

**Indirect ELISA**

Individual wells of 96 well microtiter plates (Falcon) were incubated with 100 µl of the *T. cruzi* antigen extract. The plates were incubated at room temperature overnight and then stored at −70°C until used for indirect ELISA.

Thawed plates were rinsed three times with fresh DPBS and blotted dry. Blocking solution consisting of 2.5% Carnation Instant Nonfat Dry Milk in DPBS was added to each well before incubating for one hour at 37°C. Following incubation, the plates were rinsed three times and blotted dry. Prepared murine plasma was thawed and spun for 10 minutes at 6,000 rpm. Fifty µl of supernatant was combined with 150µl of DPBS in the first well of each row creating a 1:20 dilution. Fifty µl of DPBS was added to each of the remaining wells and a 2 x serial dilution was performed across the plate. Each sample of plasma was tested in duplicate and compared with prepared normal mouse serum. Plates were covered with plastic wrap and incubated for two hours at 37°C. After incubation each plate was washed four times with DPBS. Following the fourth
wash, each well was filled with DPBS, incubated for 5 minutes, and blotted dry. Fifty μl of horseradish peroxidase-conjugated goat anti-mouse immunoglobulin (Sigma) diluted 1:1000 in blocking solution was added to each well. Plates were covered and incubated for one hour at 37° C. The plates were washed four times with DPBS following incubation and blotted dry. Fifty μl of substrate solution was added to each well and incubated for 15 minutes at 37° C. Substrate solution was prepared by dissolving 30 mg O-phenylenediamine dihydrochloride (OPD; Sigma) in 10 ml Tris Citrate Buffer (0.3 M Tris base in 0.5% Triton X-100 pH to 6.0 with anhydrous citric acid). Eight μl of 0.025% H₂O₂ was added to the substrate solution just prior to the solution being added to the wells. Plates were analyzed on a Model 550 microplate reader (Bio-Rad) set at a wavelength of 490 nm.

**Statistical analysis**

**Water consumption**

The average total water consumption of all groups per day was calculated and each group's deviation from that average was determined. Deviations were analyzed using ANOVA to determine if deviation in average water consumption per day differed significantly among groups.

**Weight**

The weight of mice before experimentation and after experimentation was analyzed using student’s paired T-test. Weight from day 28 pre was compared with
weight from day 21 pi in the paired T-test. Weight from day 21 pi was used as the ending weight because it was the last day that weight was recorded for all mice; lead-treated groups began to experience mortality on day 24 pi. Tukey’s Honest Significant Difference (HSD) test was used to analyze the initial weight of mice (day 28 pre) to determine if weight differed significantly between groups prior to experimentation. Tukey’s HSD also was used to test for difference in ending weight from day 21 pi. Weight change from day 28 pre to day 21 pi was calculated for each mouse. The difference was analyzed for significance among groups using analysis of variance (ANOVA) followed by Fisher’s Least Significant Difference (LSD) post-hoc to test for difference in multiple comparisons between groups.

*Parasitemia*

Peak parasitemia was determined for each mouse. ANOVA was used to test for significance in peaks among groups. The day on which peak parasitemia occurred was observed and analyzed by ANOVA to determine if the time in which maximum parasitemia was reached differed significantly among groups.

*Antibody concentration*

Antibody concentrations as determined by ELISA were analyzed by ANOVA using values from the third serial dilution (1:80). A significant difference was observed for antibody concentration among groups (p = 0.019, df = 4). Fisher’s LSD post-hoc was used to determine which groups differed significantly from the control. Group D (1
p.p.m.) and group E (10 p.p.m.) were shown to have significantly higher antibody concentrations from the control ($p = 0.008$, $p = 0.007$, respectively).
RESULTS

Water consumption

Analysis by ANOVA demonstrated that the deviations in volume of water consumed differed significantly between groups ($F_{4,35} = 3.335, p < 0.02$). Figure 1 shows the mean volume of water consumed per group during experimentation. The erratic patterns in water consumption demonstrate that lead-treated groups did not receive a consistent amount of lead throughout the experiment.

Survival

Figure 2 shows the percentage of surviving mice per group on day of infection beginning with day 24 pi. Mice in all lead-treated groups experienced mortality by day 28 pi, seven days earlier than the control. Groups B and E lost mice steadily throughout the experiment. Groups C and D experienced a higher rate of mortality early, stabilized, and then lost multiple mice within the last 4 days of infection. All mice were dead by day 42 pi.

Weight

Weight of mice prior to lead administration and parasitic infection did differ significantly from weight of mice exposed to lead and infected with *T. cruzi*, as shown by paired T-test ($t = -27.97$, df = 24, $p < 0.000$). Tukey's HSD analysis showed no
significant difference in weight among groups on day 28 pre ($F_{4,20} = 0.364$, $p < 0.831$). Likewise, no significant difference in weight was observed among groups on day 21 pi infection ($F_{4,20} = 0.571$, $p < 0.687$). Figure 3 shows the average weight of mice in each group until the end of experimentation. The ANOVA of weight change between day 25 pre and 21 pi showed that significant differences in weight change did exist among groups ($F_{4,20} = 4.593$, $p < 0.009$) Fisher's LSD determined that weight change from group C (0.1 p.p.m.) differed significantly from the control ($p = 0.010$). However, average weight of mice in group C was slightly lower than the control group at the onset of lead exposure (day 28 pre) and ended slightly higher (day 21 pi). Although slight, these differences in starting and ending weights were sufficient to result in a significant difference in the Fisher'sLSD analysis.

**Parasitemia**

Peak parasitemia was analyzed to determine if severity of infection differed among groups. The highest individual peak, $1.65 \times 10^7$ cells/ml of blood, was from group B, 0.01 ppm lead acetate. Although group B appeared to have a marked difference in maximum parasitemia compared to other groups (Figure 4), the difference was not significant ($F_{4,20} = 0.165$, $p < 0.953$). Most likely this variance is due to the fact that only one mouse in group B had survived to day 38 pi, the highest parasitemia occurred late in infection. The day of infection on which peak parasitemia was observed also was analyzed. A peak early in infection in a test group would denote that lead did have an adverse effect on the immune system, corresponding to increased severity of infection. No significant difference in day of peak parasitemia was found to exist among groups ($F_{4,20} = 0.242$, $p < 0.911$).
Antibody

A significant difference ($F_{4,16} = 4.056, p < 0.019$) was shown to exist between groups for concentration of antibodies produced in response to infection. Fisher’s LSD test was run on values from the third serial dilution to compare antibody concentration between groups. Group D ($p = 0.008$) and group E ($p = 0.007$) exhibited a significantly different antibody concentration from the control. In both cases the total antibody concentration was higher than that of the control. Figure 5 shows the mean antibody concentration of each group per dilution.
FIGURE 1. Average volume of drinking water consumed per group. Each group was given 200 ml’s of drinking water (either deionized or deionized plus corresponding lead acetate) at the onset of experimentation (day 28 pre). Water bottles were refilled approximately every seven days and the volume of water consumed was recorded. Day 0 represents the day of infection.
WATER CONSUMPTION

- Group A
- Group B
- Group C
- Group D
- Group E

Day of infection:
- Day 13 pre
- Day 7 pre
- Day 0
- Day 7 pi
- Day 14 pi
- Day 21 pi
- Day 28 pi
- Day 34 pi

Mean mL's consumed:
- Day 13 pre
- Day 7 pre
- Day 0
- Day 7 pi
- Day 14 pi
- Day 21 pi
- Day 28 pi
- Day 34 pi
FIGURE 2. Percent survival of test groups. Each test group began with five mice. Lead concentration in drinking water follows: group A - 0 p.p.m., group B - 0.01 p.p.m., group C - 0.1 p.p.m., group D - 1 p.p.m., and group E - 10 p.p.m.
FIGURE 3. Impact of lead acetate on mouse weight. Day 28 pre represents the beginning of the research when mice were weighed and started on lead. Day 21 pi is the last date on which all mice were alive. Weight data from day 28 pre and day 21 pi were used for statistical analysis. Day 0 represents the day of infection.
FIGURE 4. Impact of lead acetate on number of circulating parasites. Parasitemia counts began on day 14 pi and continued every 3 to 4 days until end of experimentation. Group B (0.01 p.p.m.) and group E (10 p.p.m.) each had a single surviving mouse by day 38 pi.
MEAN GROUP PARASITEMIA

- Group A
- Group B
- Group C
- Group D
- Group E

Day of infection:
- day 14 pi
- day 17 pi
- day 21 pi
- day 24 pi
- day 28 pi
- day 31 pi
- day 34 pi
- day 38 pi

Parasites per ml of blood:
- 0.00E+00
- 2.00E+06
- 4.00E+06
- 6.00E+06
- 8.00E+06
- 1.00E+07
- 1.20E+07
- 1.40E+07
- 1.60E+07
- 1.80E+07
FIGURE 5. Impact of lead acetate on antibody concentration. Serum from each test group was collected on day 32 pi and assayed along with normal mouse serum using ELISA. A 1:20 dilution was performed and data from serial dilution #3 (1:80) was used for statistical analysis.
ANTIBODY CONCENTRATION

Dilution number

Absorbance (400 nm)

1 2 3 4 5 6 7 8 9 10 11 12

- Normal Mouse
- Group A
- Group B
- Group C
- Group D
- Group E
DISCUSSION

In many previous studies, lead was shown to increase susceptibility to a number of pathological challenges. Mice have demonstrated a higher susceptibility to *Salmonella typhimurium*, encephalomyocardial virus, and *Hexamita muris*, a protozoan parasite common in mice and rats (Pelletier *et al.*, 1990). In addition, inhalation of lead chloride was shown to impair the ability of mice to eliminate bacteria from the lung. In 1996, Ellis conducted research exposing C57Bl/6 mice to lead acetate followed by infection with *Trypanosoma cruzi*. The results from her research led her to conclude that lead acetate does have immunosuppressive effects, which increases susceptibility to challenge with *T. cruzi*, thereby confirming previous results. The effects also appeared to be dose-dependent as mice exposed to higher concentrations of lead exhibited greater numbers of circulating BFT's. However, the results of the present study do not indicate that lead acetate has immunosuppressive qualities, which would increase host susceptibility to parasitic challenge.

In previous research, experimental animals were administered lead salts in drinking water. Figure 5 demonstrates the inconsistency of this method. The consumption of drinking water (and therefore lead) varied between groups and within the group over time. Therefore, lead exposure was not controlled during the course of experimentation. Mice shared a common water bottle per group; therefore no information is available on individual consumption. Lack of conclusive data may be the
result of inconsistency of lead exposure thereby requiring additional testing using a
different route for exposure.

Percent survival was the only indicator that lead does have immunosuppressive
effects as mice in all lead treated groups experienced mortality seven days earlier than
mice in the control group. However, it is unclear whether this higher mortality rate can
be attributed to increased parasitic infection resulting from lead-induced
immunosuppression or to a direct effect of the lead itself.

If lead does suppress immunological functions (thereby increasing disease
severity), one would expect to see a decrease in mouse weight, particularly in mice
receiving the highest dosages of lead (group D and group E). A significant decrease in
the weight of lead-exposed mice as compared to the weight of the control mice was not
observed. Fisher’s LSD test was used to analyze multiple comparisons. In this study, the
primary emphasis was a comparison of lead-treated mice to non-treated control mice.
However, the post-hoc test compared all groups to all other groups resulting in over
correction. Some significance may have been hidden; however, groups that were shown
to be significantly different from the control were highly significant.

In 1996, Ellis demonstrated a significant increase in mean parasitemia in mice
administered the highest lead concentrations. However, those results were not duplicated
in this experiment. An early peak in infection also would be indicative of a lower
immune response. Group D and group E, the highest lead recipients, demonstrated no
increase in parasite counts and no early peak of infection.

A significant difference among groups was found for antibody concentration. As
seen in Figure 4, mice in group D and group E have higher antibody concentrations than
mice in group A. As concentrations for both groups were significantly higher than the control, no immunological suppression of antibody production occurred.

Although each of these parameters indicates that lead does not convey suppression of the immune system and subsequent increase of susceptibility to infection, continued research is needed. The period of time in which mice ingest lead prior to infection may need to be lengthened. Koller (1973) maintained rabbits on lead for 70 days prior to pathogenic challenge. The 28 days of lead exposure prior to infection may not be enough to truly suppress the immune system. In addition, concentration of lead may need to be increased. The present research focused on lower lead dosages to determine what effect minimum exposure would have on the immune system. Previous research in which lead exposure was tested with bacterial, viral or tumor challenge maintained mice on higher lead concentrations (Koller, 1973). Route of exposure may need to be altered for lead exposure to be truly measurable and constant. An intraperitoneal injection of lead for each mouse or administering lead per os may be a solution. The lead administration would need to be maintained throughout the experiment, as some lead is lost through hair and nail growth. Other studies have been conducted using separate cages for each mouse; however, the inability for socialization may cause undue stress to the mice and could negatively impact the results. More control groups also are needed to ascertain if parameters such as mortality and weight are impacted by increased parasitic infection rather than due to a direct effect of the lead itself. Normal mouse, non-infected/lead exposed, and non-exposed/infected control groups should be added in future experiments so that interpretation of the results is more straightforward. With these suggestions in mind, future studies are planned to more fully
investigate the potential for immunosuppressive effects of lead in this experimental model of *T. cruzi* infection.
LITERATURE CITED


Ellis, T. 1996. The effect of lead acetate on host susceptibility to *Trypanosoma cruzi*. M.S. Thesis. Western Kentucky University, Bowling Green, Kentucky 75p.


Jorge, T.A., A. Bouhdidi, M.T. Rivera, M Daeron, Y. Carlier. 1993. *Trypanosoma cruzi* infection in mice enhances the membrane expression of low-affinity Fc
receptors for IgG and the release of their soluble forms. Parasite Immunology 15:539-546.


Miles, M.A. 1983. The epidemiology of South American Trypanosomiasis-biochemical and immunological approaches and their relevance to control. Transactions of the Royal society of Tropical Medicine and Hygiene 77: 5-23.


Ouaissi, M.A., A. Taibi, M. Lowens, U. Martin, D. Afchain, C. Maidana, C. Caudioti, 

Trypanosoma cruzi: A carbohydrate epitope defined by a monoclonal antibody as 
a possible marker of the acute phase of human Chagas’ Disease. American 


manifestations of metals. In Immunoxicity of Metals and Immunotoxicology, 

1993. Effect of anti-γ-interferon and anti-interleukin-4 administration on the 
resistance of mice against infection with reticulotropic and myotropic strains of 
Trypanosoma cruzi. Immunology Letters 35: 77-80.

1994. IL-10 mediates susceptibility to Trypanosoma cruzi Infection. Journal of 
Immunology 153: 3135-3140.

Reis, D., R.T. Gazzinelli, G. Gazzinelli, D.G. Colley. 1993. Antibodies to 
Trypanosoma cruzi express idiotypic patterns that can differentiate between 
patients with asymptomatic or severe Chagas’ disease. Journal of Immunology 
150: 1611-1618.

Rottenberg, M.E., M. Bakheit, T. Olsson, K. Kristensson, T. Mak, H. Wigzell, A. Orn. 
1993. Differential susceptibilities of mice genomically deleted of CD4 and CD8
to infections with *Trypanosoma cruzi* or *Trypanosoma brucei*. Infection and Immunity 61: 5129-5133.


