The Beneficial Effect of Vitamin E and Selenium During Experimental Chagas' Disease

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THE BENEFICIAL EFFECT OF VITAMIN E AND SELENIUM DURING EXPERIMENTAL CHAGAS' DISEASE

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
Yue Hou
August, 1997
THE BENEFICIAL EFFECT OF VITAMIN E AND SELENIUM DURING EXPERIMENTAL CHAGAS' DISEASE

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ACKNOWLEDGEMENTS

I wish to express my heartfelt appreciation to the following faculty members in the Department of Biology at Western Kentucky University:

Dr. Cheryl Davis, my major advisor, for assisting me with my research and thesis writing, for her tireless efforts at reading and rereading the several revisions of my thesis, answering my questions, and always being patient and eager to help me - a non-native English speaker. Her unflagging encouragement gave me the motivation to work those many, many hours which this project demanded and consumed. Her influence in my career will never be forgotten.

I would also like to thank Dr. Claire Rinehart and Dr. Sigrid Jacobshagen for their wonderful courses and for serving as my thesis committee members. I deeply appreciate the help from Dr. Doug McElroy in the statistical analysis of my data. Dr. McElroy has shown patience and support during my research and thesis writing.

Thanks to the entire faculty in the Department of Biology for their support and assistance. The two years graduate study here will become a beautiful memory in my lifetime.

Most of all, I would like to thank my husband, Xuezhi, and my two-year-old son, Yueyiqi, for their love and understanding. I do not know how I would have gotten this far without their love and support. I owe a huge debt of gratitude to my parents and my parents-in-law, for their love and unflagging support. The love and support of these family members will never be forgotten.
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THE BENEFICIAL EFFECT OF VITAMIN E AND SELENIUM DURING EXPERIMENTAL CHAGAS’ DISEASE

Yue Hou August, 1997 65 Pages

Directed by: Cheryl D. Davis, Claire A. Rinehart, and Sigrid Jacobshagen

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The present study was conducted to determine if vitamin E and selenium could have a positive synergistic effect during experimental Chagas’ disease. In the first phase of the study, 25 female C3HeB/FeJ mice were infected with a Brazil strain of Trypanosoma cruzi while receiving vitamin E supplementation of 0 IU/kg, 200 IU/kg, 400 IU/kg, 600 IU/kg, 800 IU/kg in their diets. In the second phase, 20 female C3HeB/FeJ mice were infected while receiving 400 IU/kg vitamin E plus selenium supplementation of 2 ppm, 4 ppm, 8 ppm, 16 ppm in their drinking water. The results demonstrate that vitamin E does have a protective effect during experimental Chagas’ disease. There was a statistically significant difference (p<0.05) observed between the vitamin E-deficient mice and mice supplemented with vitamin E. The results of this study also suggest that combined vitamin E and selenium supplementation provides an even greater benefit than either antioxidant alone in mice infected with T. cruzi. A statistically significant difference was observed (p<0.01) between vitamin E supplemented mice and mice supplemented with vitamin E and selenium. The beneficial effects observed included: reduced parasitemia, increased survival rate, and reduced weight loss during infection. In this study, the combination of 400 IU/kg vitamin E
and 8 ppm selenium provided the best overall protective effect during experimental Chagas’ disease.
BACKGROUND

Trypanosoma cruzi

The flagellated protozoan, Trypanosoma cruzi, causes Chagas' disease or American trypanosomiasis in humans. The parasite is distributed throughout most of South and Central America, where it infects 16 to 18 million people and kills approximately 45,000 through heart failure annually (Kirchhoff, 1993). Each year in Brazil alone, several thousand people die of heart and digestive problems brought on by the disease (Marshall, 1995). In the United States, T. cruzi has been reported from wild mammals in Maryland, Georgia, Florida, Texas, Arizona, New Mexico, California, Alabama, Louisiana (Kagan et al., 1966) and North Carolina (Karsten et al., 1992). Many kinds of wild and domestic mammals serve as reservoirs. Animals that live in proximity to humans, such as dogs, cats, opossums, armadillos, and wood rats are particularly important in the epidemiology of Chagas' disease. This disease also can be transmitted by blood transfusion (Wendel and Gonzaga, 1993). For almost 40 years, this form of transmission has been limited to Latin America, but recently, with increasing emigration to North America and Europe, Chagas' disease may be introduced to the northern hemisphere by transfusion of blood obtained from asymptomatic carriers (Kirchhoff, 1993). It has been estimated that there are 100,000 individuals living in the United States who are chronically infected with T. cruzi (Schmunis, 1991). In Argentina, Brazil, and Chile, 14 to 18 percent of new infections are
acquired from the transfusion of infected blood; and in Bolivia, approximately 49 percent of new infections are due to transfusion (Schmunis, 1991). Thus, transfusion acquired *T. cruzi* infection is a serious problem that is no longer restricted to Latin American countries.

*T. cruzi* is a stercorarian trypanosome. In its life cycle, there are three forms - trypomastigote, epimastigote and amastigote. The trypomastigote form is found in the circulating blood of the mammalian host. It is slender, 12 to 20 μm long, and its posterior end is pointed (Wendel and Gonzaga, 1993). The free flagellum is moderately long, and the undulating membrane is narrow, with only two or three undulations at a time along its length. The kinetoplast is subterminal and is the largest of any trypanosome; it sometimes causes the body to bulge around it. The amastigote stage develops in muscles and other tissues. Amastigotes are spheroid, 1.5 to 4.0 μm wide, and occur in clusters composed of many organisms (Wendel and Gonzaga, 1993). The epimastigote form is found in the gut of its insect host. Epimastigotes have a juxtanuclear kinetoplast and a flagellum (Wendel and Gonzaga, 1993).

*T. cruzi* is transmitted as an infective metacyclic trypomastigote form to mammalian hosts via the insect vector's feces. The vectors of *T. cruzi* are the hematophagous bugs of the family Reduviidae. The three genera of this family that are important in the spread of the human disease are *Panstrongylus*, *Triatoma* and *Rhodnius* (Wendel and Gonzaga, 1993). Following feeding by the vector, metacyclic trypanosomes enter the mammalian host either through the puncture wound or through nearby mucous membranes. Once inside the body, the metacyclics do not divide extracellularly but enter macrophages or
nearby tissue cells in a polarized and probably active manner (Schenkman et al., 1988), escape from the lysosomal compartment into the cytoplasm, and transform into rounded amastigotes. Intracellular division occurs several times by binary fission and the infected cells rupture. The parasites are released as tissue-derived trypomastigotes which are capable of infecting surrounding cells or disseminating to other tissues via the bloodstream. Characteristically, muscle and neuronal tissues of all types are most heavily parasitized. The \textit{T. cruzi} life-cycle is completed when blood-form trypomastigotes are taken up during feeding by the reduviid bugs. In the insect midgut, the trypomastigote become short epimastigotes, which multiply by longitudinal fission to become long, slender epimastigotes. Short metacyclic trypomastigotes appear in the insect rectum 8 to 10 days following infection (Roberts and Janovy, 1996). These infective stages can then be voided in the feces during a blood meal.

\textbf{Pathogenesis}

Chagas' disease is manifested in both acute and chronic phases. The acute phase is initiated by inoculation of trypomastigotes deposited by the feeding vector. The local inflammation produces a small red nodule, known as a chagoma, accompanied by swelling of the regional lymph nodes. In about 50\% of the cases the trypanosomes enter through the conjunctiva of the eye, causing edema of the eyelid and conjunctiva and swelling of the preauricular lymph node, known as Romana's sign (Roberts and Janovy, 1996). As the acute phase progresses, pseudocysts may be found in almost any organ of the body. The heart muscle is usually invaded, with up to 80\% of the cardiac ganglion cells being lost (Roberts and Janovy, 1996). Symptoms of the acute phase include anemia, loss of
strength, nervous disorders, chills, muscle and bone pain, and varying degrees of heart failure. Death may ensue three to four weeks after infection (Roberts and Janovy, 1996).

The acute stage is most common and severe among children less than five years old. The chronic stage; however, is most often seen in adults. The spectrum of symptoms seen in the chronic phase is primarily the result of central and peripheral nervous dysfunction, which may last for many years. Some patients may be virtually asymptomatic and then suddenly succumb to heart failure. Chagas’ disease accounts for about 70% of cardiac deaths in young adults in endemic areas (Roberts and Janovy, 1996). Part of the inefficiency in heart function is caused by loss of muscle tone resulting from the destroyed nerve ganglia. The heart itself becomes greatly enlarged and flabby.

Most infections last the life of the individual, but severity of the acute infection may determine the course of the chronic disease, possibly by affecting the type of immune response involved. Host and parasite genetic makeup, sex, age, prior infection, and a variety of other factors influence disease development and the relationship between these factors is still unresolved (Roberts and Janovy, 1996).

**Host - parasite relationship**

*Trypanosoma cruzi* can presumably infect all mammals and is transmitted to mammalian hosts in the feces of infected reduviid vectors as these insects take a blood meal. *T. cruzi* is an obligate intracellular parasite and resides most commonly within host striated muscle cells, fibroblasts and macrophages. Infection with *T. cruzi* gives rise to an immune response in humans and in other animals; this immune response is both humoral and cell- mediated (Kuhn, 1989).
A severe non-specific immunosuppression mediated by T cells and macrophages occurs following *T. cruzi* infection (Cardillo et al., 1996). In murine model systems of acute Chagas' disease, this state of immunosuppression has been shown to be associated with significant reduction of interleukin-2 (IL-2) production and suppressed expression of IL-2 receptor (IL-2R) molecules by activated lymphocytes (Majumder and Kierszenbaum, 1996).

Chronic infections are controlled primarily by humoral responses, and in some mouse/parasite strain combinations circulating IgG is protective (Roberts and Janovy, 1996). Natural killer cells and cytotoxic T lymphocytes are capable of lysing infected host cells, while activated macrophages, neutrophils and eosinophils may destroy trypomastigotes during cell-mediated responses (Kuhn, 1989).

Cytokine control is important in *T. cruzi* infection. Transforming growth factor-β (TGF-β) is activated by the parasite and has potent inhibitory effects on macrophage function (Reed, 1995). IL-10 produced by T cells, B cells and macrophages following *T. cruzi* infection downregulates macrophage activation (Reed, 1995). In contrast, Interferon-γ (IFN-γ), granulocyte macrophage colony-stimulatory factor (GM-CSF) and IL-2 protect the mammalian host from *T. cruzi* infection.

The most intimate interaction in this host-parasite relationship occurs by direct contact of the parasites with host cells; this contact, leading to subsequent internalization, is essential to the survival of the parasite. A recent study performed with cultured mammalian cells has shown that *T. cruzi* invasion is an unusual process that depends on parasite energy and on negatively charged surface molecules of the host cell (Burleigh and
Andrews, 1995). Several surface glycoproteins and mucin-like molecules of trypomastigotes were implicated primarily by inhibition studies with antibodies in interaction with host cells. Several of the trypomastigote surface glycoproteins have been shown to be related to members of a large family that includes the *T. cruzi* trans-sialidase. The mucin-like molecules are a separate family of threonine-rich, O-glycosylated molecules that function as acceptors of sialic acid in the infective stage (Burleigh and Andrews, 1995). It has been suggested that parasite surface molecules mediate binding to host cells, whereas invasion of nonphagocytic cells involves recruitment of host-cell lysosomes, an unusual event apparently triggered by signal transduction (Rodriguez et al., 1996).

**Antioxidant nutrients and disease prevention**

The possibility of a relationship in human subjects between diet and chronic or acute disease has been a major preoccupation of nutritional and medical scientists throughout a long period of history. Various constituents of food have been seen both as preventive and causative factors in the etiogenesis of disease. Activated oxygen often causes damage in living organisms and it may be the origin of a variety of diseases (Diplock, 1991). The defense of living eukaryotic cells against this damage is a complex process that involves a battery of interrelated protective agents. Antioxidant nutrients lie functionally at the heart of this protective mechanism. They include the minerals selenium, copper, zinc, and manganese, and vitamin E, C and probably A, as well as β-carotene (Diplock, 1991).

Often, as a by-product of metabolism, reactive metabolites of oxygen, such as singlet oxygen and free radicals, are formed. They can attack DNA, protein and polyunsaturated
phospholipids, and have potential detrimental effects in living systems. Therefore, it is necessary to remove such substances from the system as soon as they are formed. A free radical is defined as an atom or molecule that has one or more unpaired electron(s) (Diplock, 1991). Among various free radicals in living systems the hydroxyl radical is very highly reactive and potentially severely damaging because it can pull an electron from almost any organic molecule in its vicinity, which will initiate further radical or nonradical processes that may lie at the heart of the etiogenesis of biochemical changes that will lead to disease (Diplock, 1991). The hydroxyl radical is the product of the initial metabolites - superoxide anion and hydrogen peroxide. The control of generation of hydroxyl radical depends on the removal of these two metabolites. The superoxide anion radical is efficiently removed in the mitochondrial compartment by superoxide dismutase (SOD), which in this location is a manganese-containing enzyme, while in the cytosolic compartment the SOD is copper and zinc dependent (Diplock, 1991). Hydrogen peroxide is metabolized in both intracellular compartments by glutathione peroxidase, which is a selenium-containing enzyme (Diplock, 1991). Thus, the control of the initiation of free radical-mediated intracellular damage depends on the presence of these enzymes. In turn, the activity of these enzymes depends on the nutritional availability of the so-called antioxidant minerals, manganese, copper, zinc and selenium. Any deficiency in these nutrients may thus lower the level of the antioxidant defense mechanism so that disease processes will be initiated.

Vitamin C, vitamin E and β-carotene are important in human cellular protection because of their antioxidant functions. As with all antioxidants, there is the potential for
formation of their own radical species during the processes by which highly reactive free radicals are quenched. However, the so-called pro-oxidant forms of these substances are much less reactive and have lower energy levels than the free radicals that they neutralize (Deshpande et al., 1996). Thus, the reactivity of antioxidants in pro-oxidant form is much less than that of the harmful radicals associated with damage to tissues and organs of the body (Deshpande et al., 1996). Therefore, the antioxidant functions of micronutrients are the attributes that are most biologically relevant.

Non-antioxidant mechanisms of antioxidant vitamins also play a role in disease prevention. For example, the action of vitamin E in regulating membrane fluidity and stabilization, 5-lipoxygenase activity and protein kinase C activity are all activities independent of the capacity to scavenge free radicals (Blumberg, 1995).

Mounting evidence from laboratory and human studies points to the potential efficacy of antioxidants in reducing the risk of some cancers, cardiovascular diseases, cataracts and infectious diseases as well as injury from pro-oxidant environmental pollutants such as smog and cigarette smoke. Also, antioxidants prevent aging, inflammation and autoimmune disease (Deshpande et al., 1996).

The immune system is responsible for protection against infection by invading pathogens such as bacteria, viruses, fungi, and protozoan parasites. Cells of the immune system are highly dependent on a functioning cell membrane for secretion of lymphokines and antibodies, antigen recognition, lymphocyte transformation, and contact cell lysis. Certain cells of the immune system produce and use free radicals and reactive oxygen molecules, especially during the early stage of infection (Deshpande et al., 1996).
However, if these reactive species are over-produced during this process, they may injure the immune cells themselves as well as neighboring cells and tissues. Certain environmental factors, such as UV light, cigarette smoke and environmental pollutants can decrease immune functions. Thus, antioxidants are very necessary to keep the immune system functioning properly. Many researchers have suggested that supplementation of diets with vitamin C or E or β-carotene provides a safe and effective means to enhance clinically relevant immune functions (reviewed by Blumberg, 1995).

**Vitamin E**

Vitamin E, an essential fat-soluble vitamin, includes eight naturally occurring compounds in two classes designated as tocopherols and tocotrienols with different biological activities. The tocopherol designated as α-tocopherol has the highest biological activity and is the most widely available form of vitamin E in food (Meydani, 1995). The other isomers (β, γ, δ), some of which are more abundant in a typical western diet, are less biologically active than α-tocopherol. The commercially available synthetic forms of vitamin E are comprised of approximately an equal mixture of eight stereoisomeric forms of α-tocopherol (Meydani, 1995). Usually, the amount of vitamin E is expressed in international units (IU). Two mg of α-tocopherol is considered equivalent to 3 IU (Dutta-Roy et al., 1994).

Vegetables and seed oils are the primary sources of the tocopherols, whereas animal products are generally poor sources of this vitamin (Budowski and Sklan, 1989). Absorption of vitamin E is dependent upon digestion and absorption of fat. Free tocopherols are absorbed by a non-saturable passive process into the lymphatic circulation.
Approximately 45% of a given dose is absorbed into the lymph, while metabolites and a small amount of the intact form of vitamin E are absorbed through the portal vein. Vitamin E is transported in the blood principally by low-density lipoprotein (LDL) and high-density lipoprotein (HDL; Dutta-Roy et al., 1994). Newly absorbed vitamin E accumulates largely in adipose tissue, liver and muscle. The recommended dietary allowance (RDA) of vitamin E for men and women is 10 and 8 mg/person/day, respectively (Meydani, 1995). Relative to other fat soluble vitamins, vitamin E is safe. Few side effects have been reported, even at doses as high as 3200 mg daily.

The most widely accepted biological function of vitamin E is its antioxidant property (Dutta-Roy et al., 1994). Vitamin E is the most effective chain-breaking lipid-soluble antioxidant in the biological membrane, where it contributes to membrane stability (Dutta-Roy et al., 1994). It protects critical cellular structures against damage from oxygen free radicals and reactive products of lipid peroxidation (Deshpande et al., 1996). Vitamin E may participate in several events associated with the pathogenesis of cardiovascular disease (CVD), including LDL oxidation, adhesion of monocytes to endothelial cells, foam-cell formation and fatty-streak development, platelet adherence and aggregation, smooth muscle cell proliferation, and reperfusion injury (Meydani, 1995). The limited epidemiological studies available show an inverse relationship between dietary intake of vitamin E and risk of CVD (Meydani, 1995). Several experimental and epidemiological studies also suggest that vitamin E may reduce the risk of cancer. Vitamin E inhibits mutagenesis and cell transformation primarily through its antioxidant function, eliminating oxygen free radicals and decreasing DNA damage (Meydani, 1995).
In addition to its antioxidant function, vitamin E influences cellular responses to oxidative stress through the modulation of signal-transduction pathways (Azzi et al., 1992). Evidence from cell-culture studies (Azzi et al., 1992) indicates that when cell growth is inhibited by lipid peroxidation α-tocopherol may restimulate cell growth and proliferation by removing the inhibitor. Conversely, it may also inhibit growth of cells through its non-antioxidant properties: α-tocopherol inhibits protein kinase C activity, a signaling element that can regulate cell proliferation (Azzi et al., 1992). The specific inhibitory effect of α-tocopherol, but not of other tocopherols, on cell proliferation is dependent on the type of the cell and the stimuli used. Under oxidative stress conditions, diminution of α-tocopherol from smooth muscle cells results in growth and proliferation of these cells which could contribute to the development of atherosclerosis (Deshpande et al., 1996). The α-tocopherol modulation of signal transduction could also contribute to the anticancer and immunostimulatory effects of vitamin E (Meydani, 1995).

Reproductive failure in the rat was the first vitamin E deficiency syndrome described (Evans and Bishop, 1922). Anemia is a characteristic symptom in vitamin E-deficient monkeys (Fitch, 1968) and piglets (Baustad and Nafsted, 1972). Deficiency diseases in human adults caused by insufficient intake of dietary vitamin E are extremely rare because of the ubiquitous distribution of the vitamin. However, two groups of individuals are susceptible to severe hypovitaminosis E. These are neonates and patients with disorders of fat absorption or transport (Budowski and Sklan, 1989).

In chronic liver diseases, chronic cholestasis, cystic fibrosis and ileal resection, circulating tocopherol levels are usually very low (Budowski and Sklan, 1989). Patients
develop neurological symptoms, sometimes accompanied by muscular weakness. There is strong evidence that vitamin E deficiency is responsible for this neuropathy, and massive oral doses or intramuscular administration of vitamin E have been shown to relieve the neural symptoms (Muller et al., 1983; Nelson, 1983). It appears that vitamin E deficiency in humans primarily affects the central and peripheral nervous systems.

The cells of the immune system are highly susceptible to the harmful effects of free-radical reactions. Vitamin E, as the major vitamin antioxidant in plasma and tissue, protects immune cells from these harmful effects. Deficiency in vitamin E is associated with a decline in the immune response, whereas higher than recommended levels of vitamin E have stimulatory effects on the immune system. In a double-blind study examining the effects of vitamin E on immune responses, Meydani et al. (1989) found significant improvement in several clinical indicators of immune function in healthy subjects 60 years of age and older. Studies of murine AIDS suggest that vitamin E supplementation acts on various immune components to modify immune defects induced by retrovirus infection and that vitamin E effectively alleviates nutrient deficiencies induced by retrovirus infection in the immune organs (Wang et al., 1994). High doses of vitamin E were shown to increase humoral antibody production against particulate and soluble antigens in different animal species and to enhance phagocytosis (Tengerdy, 1980). Cell-mediated immune function was also stimulated by high dietary levels of vitamin E in several experimental animals (Corwin and Schloss, 1980; Bendich et al., 1983; 1984; 1986). The beneficial effects of vitamin E on immune function may be attributed to the inhibition of the production of certain immunosuppressive prostaglandins (Tengerdy,
1980) or moderation of granulocyte activation (Deshpande et al., 1996). The protective
effect of vitamin E, therefore, may be associated with a reduction in the $H_2O_2$ generated by
phagocytic cells of the immune system (Deshpande et al., 1996).

**Selenium**

Selenium is a component of several enzymes, in particular those related to redox
reactions. It was found to be an integral part of the antioxidant enzyme, glutathione
peroxidase, which protects cells by reducing intracellular peroxides (Spallholz, 1994). As
an antioxidant mineral, both deficiency and excess were found to have deleterious effects
on several animal body systems, including the immune system (WHO, 1987). Dietary
sources of selenium include cabbage, celery, radishes, brewer’s yeast, fish, whole grains,
and meat. Selenium is present in organic form in these sources and the organic form is the
major nutritional source of selenium for animals and humans (Madhavi et al., 1996).

Ricettl and coworkers (1994) reported the effects of selenium (Se) supplementation
on glutathione peroxidase (GSH-Px) activity, on prostacyclin (PGI$_2$) production, and on
GSH-Px mRNA expression in cultured human umbilical vein endothelial cells (HUVEC).
Se-enriched HUVEC showed a significant increase in both GSH-Px activity and thrombin-
stimulated production of PGI$_2$. On the other hand, an inverse correlation was observed
between Se concentrations in culture media and GSH-Px mRNA levels in Northern blot
analysis. These results suggested that a major degree of regulation for GSH-Px expression
by Se was most likely exerted at the post-transcriptional level. Selenoprotein P is another
plasma selenoprotein and is a major participant in selenium metabolism (Burk and Hill,
1994). When the supply of selenium is limiting, selenoprotein P synthesis has priority over
glutathione peroxidase synthesis (Burk and Hill, 1994). Selenoprotein P has been purified from human plasma. In selenium deficient humans, selenoprotein P concentrations are low (Burk and Hill, 1994). The proposed functions of selenoprotein P are in oxidant defense and in the distribution of selenium throughout the body. However, the possibility of antioxidant protection provided directly by selenoprotein P must be further investigated.

Selenium deficiency is associated with the development of two diseases, both of which are only seen in China where acutely low soil levels of the selenium are reflected in very low blood levels. Keshan Disease is a cardiomyopathy of children and young adults (Xia et al., 1989), and Kachin Bech disease is an osteoarthropathy that occurs primarily in young people (WHO, 1987). Both diseases are preventable by therapy with selenium supplementation. But several features of the diseases suggest that they are not simple selenium deficiency diseases, rather, they are multifactorial in origin, and selenium deficiency is a major factor in their etiology (WHO, 1987).

There is a growing body of strong epidemiological evidence that shows a correlation between low dietary intake of selenium and an increased risk of some cancers. A recent study showed that selenium, but not other antioxidants, may help protect against ovarian cancer (Stenson, 1996). In another study, the effect of selenium on murine tumor formation was observed; the results showed that selenium was anticarcinogenic both in vivo and in vitro. Dietary supplementation with 2.0 ppm selenium markedly inhibited DMBA-induced mammary carcinogenesis, and this level of selenium was not significantly detrimental to the normal physiological functions of the host (Medina et al., 1983). The mechanisms involved in the protective effect of selenium in mammary tumorigenesis may
be at the level of DNA synthesis because selenium exposure leads to DNA fragmentation and increased mutagenesis in tumor cells. This effect is not attributable to GSH-Px activity.

The effects of selenium deficiency have been studied in bacterial, yeast, parasitic and viral infections. Selenium deficiency impaired the ability of mouse neutrophils to kill Candida albicans in vitro, and thus selenium deficient mice were susceptible to C. albicans (Boyne and Arthur, 1986). In contrast, selenium deficiency was shown to increase the resistance of rats to Salmonella typhimurium (Boyne et al., 1984) and the resistance of mice to Plasmodium bergeii, Listeria monocytogenes and Pseudorabies virus (Boyne and Arthur, 1986).

A number of studies have documented that the availability of selenium in the diet affects the immune functions of a host in vivo and that selenium deficiency and selenium supplementation correlate, respectively, with a decreased or increased response of a host to challenge with foreign antigens.

HIV infection results in poor oral uptake and gastrointestinal malabsorption, which results in selenium deficiency. Diminished levels of Se are seen in both blood and tissue (Dworkin, 1994). Patients with AIDS tend to have a more severe deficit in Se than those with earlier stages of HIV infection. It has been suggested that in patients with AIDS, selenium deficiency may be associated with myopathy, cardiomyopathy and immune dysfunction including oral candidiasis, impaired phagocytic function and decreased CD4+ T-cells (Dworkin, 1994). Thus, Dworkin (1994) has suggested that selenium along with other nutrients must be considered in the nutritional therapy of AIDS patients.
Selenium supplementation significantly increased immunoglobulin M and G anti-sheep red blood cell hemagglutinating titers in Swiss Webster mice while Se toxicity or Se deficiency resulted in titers approximately equal to or less than control mice (Spallholz et al., 1973). Roy et al. (1994) studied the effect of supplementation with selenium on human immune cells. Dietary supplementation of Se-replete humans with 200 ug/day of sodium selenite for 8 wk, or in vitro supplementation with $1 \times 10^{-7}$ M Se (as sodium selenite), resulted in a significant augmentation of the ability of peripheral blood lymphocytes to respond to mitogen or alloantigen and to express high affinity IL-2 receptors (IL-2R) on their surface. Supplementation with Se could apparently modulate T-lymphocyte-mediated immune responses in humans that depend on signals generated by the interaction of IL-2 with IL-2 R. Also, Se supplementation resulted in a 118% increase in cytotoxic lymphocyte-mediated tumor cytotoxicity and an 82.3% increase in natural killer cell activity as compared to baseline values (Kiremidjian-Schumacher et al., 1994). This result was also correlated with the expression of high affinity IL-2 R, and consequently the rate of cell proliferation and differentiation into cytotoxic cells was enhanced. The results indicated that the immunoenhancing effects of selenium in humans require supplementation above levels produced by normal dietary intake. In a follow-up study, Roy and coworkers (1995) demonstrated that supplementation with selenium resulted in a significant increase in the ability of splenic lymphocytes from aged male C57Bl/6JNIA mice to undergo blastogenesis, as indicated by significantly higher amounts of nuclear incorporation of $^3$H-thymidine after stimulation with mitogen. It was also found that dietary supplementation with Se restored the age-related deficiency of the cells to respond
to mitogen stimulation by nuclear DNA synthesis and cell proliferation, at least to the level of cells from unsupplemented young adult mice. Roy et al. (1995) also studied the effect of Se \textit{in vitro}, and the results showed that alloantigen-activated lymphocytes from Se-supplemented aged mice contained significantly higher numbers of cytotoxic lymphocytes than those from Se-normal aged mice, which resulted in an enhanced capacity to destroy tumor cells. This study verified again that the increased number of high-affinity IL-2R resulted in cell proliferation when Se restored the age-related defect in immune cell function.

\textbf{The combined effect of vitamin E and selenium}

As antioxidants, either vitamin E or selenium can protect fully against certain diseases in animals such as liver necrosis in rats or exudative diathesis in chicks (Hoekstra, 1975). However, selenium cannot substitute for vitamin E in some other conditions, such as fetal resorption in rats or encephalomalacia in chicks. It is thought that even under conditions of adequate dietary selenium intake, glutathione peroxidase could not sufficiently protect the target tissues (Levander, 1992). Selenium and vitamin E in particular appear to have overlapping and synergistic functions.

Williams et al. (1994) studied the interaction of vitamin E and selenium in mice resistant to virus-induced myocarditis. C57Bl/6J mice fed a normal diet were resistant to coxsackievirus B\textsubscript{3} (CVB\textsubscript{3}) induced myocarditis. In this study, C57Bl/6J mice were fed one of four experimental diets. After consuming the diets for 4 weeks, mice were inoculated with a myocarditic strain of CVB\textsubscript{3}. Ten days post inoculation, mice fed diets deficient in both Se and vitamin E developed myocardial lesions consisting of an inflammatory
infiltrate. Mice fed adequate diets or diets singly deficient in either Se or vitamin E did not develop myocarditis. Mice fed the double-deficient diet showed more severe myocarditis than singly deficient-fed mice by developing glucosuria and elevated heart viral titers. These results suggested an interaction of dietary vitamin E and selenium in determining the outcome of infection with CVB₃.

Murine malaria appears to be a useful experimental model for investigating interrelationships of selenium and vitamin E because the malarial parasite is highly susceptible to oxidative stress. In a study performed by Levander (1992), vitamin E deficiency was found to protect against the parasitic infection while selenium deficiency had little or no protective effect against the parasite. This study indicated that nutritional manipulation of host antioxidant status may provide a promising prophylactic and/or therapeutic tool for the control of malaria.

In a study of experimental diabetes in rats, the combination of selenium and vitamin E showed an optimal effect in the delay of nephropathy (Douillet et al., 1996). Three groups of diabetic rats were supplemented either with a Se-rich yeast diet (selenion), or with selenomethionine, or with a double supplementation with selenomethionine and vitamin E. These treatments led to an increase in selenium levels in plasma in the three groups as compared with the unsupplemented diabetic group or the control group without disease. These investigators concluded that combined treatment with Se and vitamin E provided the most beneficial effect in protecting kidneys in diabetic rats with expression in a significant correction of renal hyperfiltration and in a diminution of the number and severity of glomerular lesions.
Vitamin E and selenium have immunostimulatory effects in a variety of species when administered in quantities in excess of established dietary requirements (Sheffy and Schultz, 1979). Conversely, deficiencies in vitamin E and selenium lead to suppression of immune responses, particularly cell mediated mechanisms. The responses to each of the nutrients appear to be independent.

A combined deficiency in vitamin E and selenium in young chickens resulted in histopathology of primary lymphoid organs, depressed humoral response to T cell-dependent antigens, reduced mitogenic responses of lymphocytes and impaired macrophage function (Dietert et al., 1990). Further studies of cell surface marker expression in chickens showed that vitamin E and selenium deficiency may negatively affect both the maturation of specific lymphocyte subpopulations and the functional and proliferative capabilities of the peripheral lymphocytes (Chang et al., 1994).

In a study of channel catfish (Wise and Tomasso, 1993), intracellular superoxide anion production of macrophages was higher in fish fed a diet fortified with four times the recommended levels of both vitamin E and selenium than in fish fed other diets (a diet deficient in both nutrients; a diet deficient in either Se or vitamin E; and a diet adequate in both nutrients). These results indicated that Se and vitamin E could not compensate for each other and that the combined supplementation with both antioxidants greatly enhanced macrophage function.

Dietary supplementation of mammals with vitamin E and Se is important in the maintenance of host defense mechanisms, including antibody production, cell proliferation, cytokine production, prostaglandin metabolism, and neutrophil function (Deshpande et al.,
Hogan et al. (1993) reported that there was a synergistic effect of vitamin E and Se on the duration of clinical mastitis in cows. Cows supplemented with both vitamin E and Se experienced a shorter duration of clinical signs than cows supplemented with either nutrient alone. Neutrophils are very important in the host defense against bovine bacterial infection. Dietary supplementation with vitamin E and selenium successfully optimized neutrophil responses resulting in increased resistance to the bacteria which caused mastitis.

The effect of vitamin E and selenium on the immune response also has been investigated in sows (Wuryastuti et al., 1993). The results of this study showed that the phagocytic ability of colostral polymorphonuclear (PMN) cells was diminished significantly only in the cells from sows deficient in both vitamin E and selenium. The ability of peripheral blood PMN to engulf yeast cells in vitro was impaired by both vitamin E and Se deficiencies and was impaired sooner by the combined deficiency than by individual deficiency.
INTRODUCTION

During the past two decades, global epidemiological studies have been targeted at uncovering the causes of several important diseases afflicting humans. These studies have clearly demonstrated that many diseases, especially chronic diseases that affect humans, have an uneven geographic distribution (Weisburger, 1991). Economics or local dietary habits are often important contributing factors leading to the uneven geographic distribution of these diseases. Epidemiological studies linking the prevalence of certain diseases to dietary patterns often tend to show an inverse correlation between the consumption of certain foods and the occurrence of certain diseases (Deshpande et al., 1996).

Chagas' disease is a severe parasitic disease that is prevalent throughout Central and South America. At the present time it is regarded as a major cause of morbidity and mortality in tropical areas, particularly among the poor in developing nations (Kirchhoff, 1993). Many people in these areas are undernourished or malnourished (Foster, 1992), and many children (especially those under the age of five) die of diseases either caused or complicated by undernutrition (Foster, 1992). Generally speaking, a sufficient diet is important for human health, especially for children and young adults because of their rapid growth rate. Acute Chagas' disease is most severe in children under five years of age, and most new infections in rural areas occur in young children (Kirchhoff, 1993).
Although Chagas' disease is not caused by malnutrition, the uneven distribution of this disease in Latin America suggests that nutrition and other socioeconomic factors may play a role in this vector-borne parasitic infection.

Antioxidants have been studied for many years, and their function in disease prevention has been recognized. The antioxidant activities of vitamin E and selenium in particular have been studied in a variety of species (Hoekstra, 1975; Levander, 1992). At the biochemical level, primary defenses against free radical mediated cell damage involving the major enzyme systems and their substrates are supported by secondary defenses. Selenium is an essential nutrient with antioxidant properties and is necessary for the synthesis and activity of glutathione peroxidase, a primary cellular antioxidant enzyme in the primary defense (Deshpande et al., 1996). Vitamin E is the principle component of the secondary defense against free radical mediated cellular injuries. In fact, it is the only natural, physiological, lipid-soluble antioxidant that can inhibit lipid peroxidation in cell membranes (Budowski and Sklan, 1989). Thus, vitamin E and selenium play separate but interrelated roles in the cellular defense against oxidative damage. Also, both vitamin E and selenium have been shown to have immunostimulatory effects in a variety of species when administered in quantities in excess of established dietary requirements (Sheffy and Schultz, 1979). Deficiency in or supplementation with these two antioxidants has been shown to affect the course of a variety of diseases or to have some clinical therapeutic effects.

Studies of host cell invasion by *Trypanosoma cruzi*, the etiological agent of Chagas' disease, have suggested that macrophages in host tissue play an important protective role
against the parasite (Burleigh and Andrews, 1995). Activated macrophages are able to kill intracellular trypomastigotes or amastigotes by toxic oxygen intermediates and nitric oxide. However, the respiratory burst and the production of nitric oxide by macrophages also may cause damage to surrounding host cells. As antioxidants, vitamin E and selenium can help to protect cells from the damage caused by reactive oxygen intermediates. Under conditions of normal nutrition, vitamin E and selenium levels in the diet should be adequate to provide a certain level of protection against reactive oxygen intermediates generated during *T. cruzi* infection. However, it seems likely that individuals who subsist on diets deficient in one or both of these antioxidants may be more susceptible to the severe pathological consequences of Chagas’ disease. Vegetable and seed oils are the primary nutritional source of vitamin E (Deshpande et al., 1996), while meat products are good dietary sources of selenium (WHO, 1987). In the poor area of Latin America, where typical diets may be lacking in these kinds of foods, individuals may well be deficient in vitamin E or selenium as well as other antioxidant micronutrients. It is, therefore, of interest to determine if there is a protective effect associated with dietary supplementation with vitamin E and selenium during infection with *T. cruzi*.

The present study was designed to determine the combined effect of dietary supplementation with different doses of vitamin E and selenium during experimental Chagas’ disease. The effect was observed by measuring weight, parasitemia, antibody production and mortality. The optimal combination dose of vitamin E and selenium was also determined.
MATERIALS AND METHODS

Parasites

The Brazil strain of *Trypanosoma cruzi* was used in all experiments. The parasites were maintained by serial passage into C3HeB/FeJ mice at 3 week intervals. An infection inoculum of $1 \times 10^3$ blood-form trypomastigotes (BFTs) in 0.2 ml of sterile Dulbecco’s phosphate buffered saline (DPBS; Sigma Chemical Co., St. Louis, Missouri) was administered intraperitoneally to each mouse.

Mice and diets

In the first phase of the study, 25 female C3HeB/FeJ mice (Jackson Laboratories, Bar Harbor, Maine) were maintained at room temperature (26°C). The mice were five-six weeks old upon arrival and were immediately placed on one of the diets described below. Pelleted food and distilled water were provided *ad libitum*. The mice were randomly separated into five cages of 5 mice each and were given food containing varying amounts of vitamin E (Vit E) without selenium as follows: Cage 1 - 0 IU/kg Vit E; Cage 2 - 200 IU/kg Vit E; Cage 3 - 400 IU/kg Vit E; Cage 4 - 600 IU/kg Vit E; Cage 5 - 800 IU/kg Vit E (Purina Mills, Richmond, Indiana). Mice were infected three weeks after being placed on the experimental diets.

In the second phase of the study, 20 female C3HeB/FeJ mice (5-6 weeks of age) were randomly separated into four cages of 5 mice each and were given food containing
400 IU/kg Vit E (optimal dose determined from the first phase of the study). Drinking water containing different doses of sodium selenate (Se; Sigma Chemical Co.) was given as follows: Cage 1 - 2 ppm Se; Cage 2 - 4 ppm Se; Cage 3 - 8 ppm Se; Cage 4 - 16 ppm Se. Mice were infected three weeks after being placed on the experimental diets. Food and drinking water were provided *ad libitum*.

**Parasitemia**

Parasite counts were conducted twice weekly beginning on day 12 post-infection. A 4 μl sample of blood was taken from the tail of each mouse and was diluted with 96 μl DPBS to make a 1/25 dilution. A hemacytometer (Fisher Scientific, Pittsburgh, Pennsylvania) was used to count the parasites.

**Weight and mortality**

The mice were weighed on the first day of arrival and on the day before they were infected with *T. cruzi*. Mice were then weighed on days 14, 28 and 42 of infection.

Mortality was monitored throughout the course of the experiments.

**Trypomastigote antigen preparation**

Fibroblast-derived trypomastigotes of the Brazil strain were cultured in PSC3H murine fibroblasts (Gooding, 1977) infected with BFTs of *T. cruzi*. The cells were cultured at 36°C in RPMI 1640 medium (HEPES modification; Sigma Chemical Co.) adjusted to pH 7.2 and supplemented with penicillin G (100 units/ml), streptomycin (100 mg/ml) and 10% fetal bovine serum in a 5% CO₂ incubator. Culture supernatants containing trypomastigotes were collected and filtered through Whatman #1 filter paper (Jacobson et al., 1992). The filtered supernatants were then centrifuged for 20 minutes at
1800 x g. The pellet, containing parasites, was resuspended in 1 ml of DPBS, and centrifuged for 5 minutes at 5000 x g. Following 2 additional washing steps, the pellets were combined and resuspended in ice-cold DPBS containing 0.5% Triton X and 2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.). Parasite lysis and detergent solubilization were achieved by frequent vortexing during a 30 minute incubation period on ice. The resulting suspension was spun at 5000 x g for 15 minutes to remove insoluble material and the supernatant was collected. A Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, California) was performed to determine the protein concentration of the antigen preparation using bovine serum albumin as a standard. The antigen extract was diluted to a working concentration of 25 μg/ml in DPBS for use in an indirect enzyme linked immunosorbent assay (ELISA).

**Parasite-specific antibody levels**

Individual wells of 96 well microtiter plates (Falcon Micro Test III; Becton Dickinson, Oxnard, California) were incubated with 50 μl of parasite antigen (25 μg/ml) at room temperature overnight and were then stored at -70°C until use. All mice were bled on days 20 and 35 post-infection. A 20 μl sample of blood was taken from the tail of each mouse and was diluted in 180 μl of DPBS. Diluted blood was then centrifuged for 10 min at 8,000 x g and the supernatant was removed and stored at -70°C until use. Normal mouse plasma, prepared in the same manner, was used as a control in the enzyme-linked immunosorbent assay (ELISA). The *T. cruzi* antigen-coated plates were blocked for 1 h at room temperature with 2.5% nonfat dry milk (Carnation Co., Los Angeles, California) in DPBS. Following the blocking step, the plates were washed 3 times in DPBS. A 100 μl
aliquot of the plasma sample for each infected mouse was added to the first well of each row of the plate in duplicate and a two-fold serial dilution (in DPBS) of the plasma was performed across each row. The dilution of plasma in the first well of each row was 1/55. The plate was incubated for 2 h at 37°C. Following 4 washes in DPBS, 100 μl of the goat anti-mouse immunoglobulin (IgG, IgM, IgA) conjugated to horseradish peroxidase (Sigma Chemical Co.) was placed in each well of the plate at a dilution of 1:1000 in the blocking solution and the plate was incubated for 1 h at 37°C. After a final washing step in DPBS, the substrate O-phenylenediamine (Sigma Chemical Co; 30 mg), dissolved in 11.5 ml of Tris-citrate buffer (pH 6.0) and activated with 30% hydrogen peroxide (H₂O₂; 10 μl in 1 ml H₂O), was added to all wells of the plate (50μl/well). The absorbance of each well of the 96 well microtiter plate was measured at a wavelength of 490 nm with a Model 550 microplate reader (Bio-Rad Laboratories). Antibody titers were determined according to the following equation: titer = highest dilution of the test plasma in which the absorbance is > 0.05 and > the mean control absorbance + 2 standard deviations (SD).

**Statistical analysis**

Data were analyzed using SYSTAT (SYSTAT, 1992). Canonical variates analysis (CVA) was used to test for differences among Vit E treatment groups in the first phase of the study and among groups given different doses of selenium in conjunction with an optimal dose of Vit E in the second phase of the study. Variables included in CVA are Peakday, Firstday, Meanpeak, Peakvalue, Antibody, Weight 1, Weight 14, Weight 28. Peakday refers to the day on which individual mice have the highest number of parasites in the peripheral circulation. Firstday refers to the first day on which parasites were detected
in the blood of individual mice. **Meanpeak** refers to the number of parasites on the day that was the average value of the Peakdays of all mice in one treatment group. **Peakvalue** refers to the highest number of parasites in the blood of individual mice. **Antibody** refers to the absorbance of individual plasma samples in the first dilution (1/55) of ELISA. **Weight 1, 14, 28** refers to weight of individual mice on the day before infection, day 14 and day 28, respectively. To separate effects of Vit E deficiency from those of different doses of Vit E, two CVA, one including all Vit E treatment groups and the other excluding Vit E deficient group, were conducted in the first phase of the study. The 0 ppm Se plus 400 IU/kg Vit E treatment group in the first phase of the study served as the Se-deficient control group for the second phase of the study. Mortality data could not be included in the CVA because only one value (i.e., percent survival) is obtained for each treatment group.
RESULTS

Effect of vitamin E supplementation on weight

The weights of all mice were measured on days 14 and 28 post-infection and the results are shown in Figure 1. Mice in the Vit E deficient group showed the greatest loss of average weight among all groups from day 14 to day 28 of infection. The percent loss per kilogram average weight for this group was nearly two fold greater than that of any Vit E supplemented group (Table 1a; 23.15% versus 8.7-12.20%). Among Vit E supplemented groups, the 800 IU/kg Vit E treatment group showed the least average loss of weight followed by the 400 IU/kg Vit E treatment group. However, as shown in Figure 1, the data for the 800 IU/kg Vit E treatment group showed a larger standard deviation than the 400 IU/kg Vit E treatment group. The average weight on day 42 of infection is not shown due to mortality.

Effect of vitamin E supplementation on parasitemia

Mean parasitemia levels for each treatment group of mice are shown in Figure 2. The 400 IU/kg and 600 IU/kg Vit E treatment groups had lower parasitemias than the other three Vit E treatment groups, while the 800 IU/kg Vit E treatment group showed the highest parasitemia levels. Parasitemia values for the vitamin E deficient group were intermediate between the parasitemia values observed for the 200 IU/kg Vit E treatment group and the 400 IU/kg Vit E treatment group.
Effect of vitamin E supplementation on mortality

Dietary supplementation with Vit E (even in the absence of selenium) resulted in increased longevity and survival in infected mice. As shown in Figure 3, despite only moderately high parasitemia levels, the Vit E deficient group showed 100% mortality by day 43 post-infection. In contrast, by day 48 of infection the 400 IU/kg treatment group had the highest survival rate (80%) followed by the 600 IU/kg treatment group which had a survival rate of 40%. On day 53 of infection, the 400 IU/kg Vit E treatment group showed a survival rate of 60%, while the 600 IU/kg and 800 IU/kg Vit E treatment groups showed 20% and 0% survival, respectively. At the end of the first phase of the study (day 70 post-infection), the 400 IU/kg and 600 IU/kg Vit E treatment groups showed 20% survival while all other treatment groups exhibited 0% survival.

Parasite-specific antibody response in phase I

Antibody levels for each Vit E treatment group are shown in Figure 4. Antibody levels were similar for all treatment groups, although mice in the 600 IU/kg Vit E treatment group showed the highest absorbance values at all dilutions in the assay on day 20 of infection. There was no statistically significant difference in parasite-specific antibody levels between different Vit E treatment groups on day 20 or on day 35 of infection (p > 0.05). Parasite-specific antibody titers obtained from ELISA results are listed in Table 2a and 2b. The highest antibody titer was observed in the 600 IU/kg Vit E treatment group on day 20.

Statistical analysis in phase I

The overall effect of the Vit E treatments differed significantly among groups (F_{32, 12}
Vit E deficient mice were significantly different from all Vit E supplemented groups (Test of residual roots $X^2 = 65.75$, df = 32, p < 0.001). There was no statistically significant difference among Vit E supplemented groups (Test of residual roots $X^2 = 28.98$, df = 21, p = 0.114). Weight 1 and Weight 14 load heavily on canonical variate I (Fig. 5a) and thus are major contributors to the differences among treatment groups on that axis. The Vit E supplemented groups had lower weights than the Vit E deficient group on both the day before infection and on day 14.

**Effect of selenium and vitamin E supplementation on weight**

Weights of all mice were measured in the second phase of the study on days 14, 28, and 42 of infection. Figure 6 shows that the 0 ppm Se treatment group had the greatest loss of average weight among all treatment groups from day 14 to day 42 of infection. The percent loss per kilogram average weight of this group was at least 3 fold greater than that of any Se supplemented group from day 14 to day 42 (Table 1b; 28.05% versus 0.95-8.91%). Among the Se supplemented groups, the 8 ppm Se treatment group showed the least loss of average weight from day 14 to day 42 of infection. The 2 ppm and 8 ppm Se treatment groups actually showed a percent weight gain between day 14 and day 28 of infection.

**Effect of selenium and vitamin E supplementation on parasitemia**

Parasitemia levels measured in the second phase of the study are shown in Figure 7. The 8 ppm Se plus 400 IU/kg Vit E treatment group had the lowest mean parasitemia (1.44×$10^6$/ml) at its peak. In contrast, the Se deficient group exhibited the highest peak parasitemia (2.72×$10^6$/ml) among all treatment groups. The second highest peak
parasitemia \((2.45 \times 10^6/\text{ml})\) was observed in the 16 ppm Se treatment group.

**Effect of selenium and vitamin E supplementation on mortality**

Survival rates in the second phase of the study are shown in Figure 8. The 0 ppm Se treatment group showed the lowest overall survival rate of 20%, whereas the 8 ppm and 16 ppm Se treatment group showed the highest survival rate of 80% by the end of the second phase of the study (day 70 post-infection). Combined treatment with 400 IU/kg Vit E plus 8 ppm Se or 16 ppm Se increased the survival rate of infected mice 4 fold above that observed when mice were given 400 IU/kg Vit E in the absence of Se in the first phase of the study.

**Parasite-specific antibody response in phase II**

The parasite-specific antibody levels for all Se plus Vit E treatment groups on day 35 of infection are shown in Figure 9. The antibody titers are given in Table 2c. Mice treated with 400 IU/kg Vit E and Se showed antibody titers at least 4 fold higher than observed on the same day of infection in the 400 IU/kg Vit E (0 ppm Se) treatment group. However, statistical analysis indicated that there was no significant difference in the parasite-specific antibody levels among different Se treatment groups. Compared with the antibody data obtained in the first phase of the study on day 35 of infection, parasite-specific antibody titers of all but 16 ppm Se plus 400 IU/kg Vit E treatment groups were at least 2 fold greater than observed in groups given only Vit E (Table 2b, 2c). Despite these observed differences in antibody titer, there was no statistically significant difference between Vit E plus Se treatment groups and the groups given Vit E only.
Statistical analysis in phase II

The overall effect of Se plus 400 IU/kg Vit E treatment showed a statistically significant difference among treatment groups ($F_{40, 28} = 2.898, p = 0.002$). Both canonical variate I (Test of residual roots $X^2 = 77.05, df = 40, p < 0.001$) and canonical variate II (Test of residual roots $X^2 = 43.94, df = 27, p = 0.021$) showed significant differences among treatment groups. Weight 1, 14, and 28 were the primary contributing factors to separation of groups on canonical variate I (Fig. 10). Meanpeak, Weight 14 and Weight 28 were main contributing factors in determining significant differences among Se treatment groups on canonical variate II. Mice in the 16 ppm Se treatment group showed the lowest weights on the day before infection and on days 14 and 28 of infection, followed by mice in the 8 ppm Se treatment group. The 0 ppm Se supplemented group showed the highest weights on the day before infection and on days 14 and 28. On canonical variate II, the 4 ppm Se treatment group showed lower levels of parasites in the blood and higher weights on days 14 and 28 of infection, followed by the 16 ppm and 8 ppm Se supplemented groups.
TABLE 1. Weight loss of *T. cruzi*-infected mice with vitamin E supplementation (a) or supplementation with selenium plus vitamin E (b). The loss of weight per kilogram average body weight from day 14 to day 28 or from day 14 to day 42 was calculated for each vitamin E treatment group (all with 0 ppm selenium) or each selenium plus vitamin E (400 IU/kg) treatment group.

a.

<table>
<thead>
<tr>
<th>Vit E Treatment (IU/kg) with 0 ppm Se</th>
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<td>% Loss day 14-28</td>
<td>23.15</td>
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b.

<table>
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<th>Se Treatment (ppm) with 400 IU/kg Vit E</th>
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<th>8</th>
<th>16</th>
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<tr>
<td>% Loss day 14-28</td>
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<td>% Loss day 14-42</td>
<td>28.05</td>
<td>8.21</td>
<td>8.91</td>
<td>0.95</td>
<td>3.42</td>
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</table>
TABLE 2. Parasite-specific antibody titers. a) Data are presented for vitamin E treatment groups (all with 0 ppm selenium) on day 20. b) Data are presented for vitamin E treatment groups (all with 0 ppm selenium) on day 35. c) Data are presented for selenium plus vitamin E (400 IU/kg) treatment groups on day 35.

<table>
<thead>
<tr>
<th>a.</th>
<th>Vitamin E Treatment (IU/kg)</th>
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<th>c.</th>
<th>Selenium (ppm) with 400 IU/kg Vit E</th>
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<tr>
<td></td>
<td>Titer</td>
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<td>1/28160</td>
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FIGURE 1. Effect of vitamin E supplementation on weight of *T. cruzi*-infected mice. The average weight of individual *T. cruzi*-infected mice in each vitamin E treatment group (all with 0 ppm selenium) was calculated on days 14 and 28 post-infection, respectively. Data are presented as average weight + standard deviation of weights of five mice in each group.
FIGURE 2. Effect of vitamin E supplementation on parasitemia of *T. cruzi*-infected mice.

Points represent the mean number of blood-form trypomastigotes per milliliter of blood (five mice per group; all with 0 ppm selenium). Standard deviations are not indicated to preserve the clarity of the figure.
Day Post-Infection

Mean Number of Parasites/ml (x10000)

- ▲ 0 IU/kg Vit E
- ▲ 600 IU/kg Vit E
- △ 200 IU/kg Vit E
- ▲ 800 IU/kg Vit E
- ○ 400 IU/kg Vit E
FIGURE 3. Effect of vitamin E supplementation on mortality of *T. cruzi*-infected mice.

The percent survival for each vitamin E treatment group (all with 0 ppm selenium) is presented for different days post-infection.
Day Post-Infection

- 0 IU/kg Vit E
- 600 IU/kg Vit E
- 200 IU/kg Vit E
- 800 IU/kg Vit E
- 400 IU/kg Vit E
FIGURE 4. Plasma levels of parasite-specific antibody on day 20 (a) and on day 35 (b) post-infection in mice infected with *T. cruzi*. All mice were treated with varying doses of vitamin E without selenium. Levels of anti-*T. cruzi* antibodies were measured in unpooled plasma collected from individual infected mice and pooled normal mouse plasma. Data are presented as the mean absorbance (at 490 nm) of five plasma samples in each vitamin E treatment group. Data obtained from normal mouse plasma are presented as the mean absorbance ± 2 standard deviations of duplicate values.
a

![Graph showing the relationship between absorbance and reciprocal dilution for different concentrations of Vitamin E. The graph includes data for 0 IU/kg, 200 IU/kg, 400 IU/kg, 600 IU/kg, and 800 IU/kg of Vitamin E.]
Reciprocal Dilution (x10)

Absorbance

- ▼ NMP
- □ 0 IU/kg Vit E
- ▲ 200 IU/kg Vit E
- ○ 400 IU/kg Vit E
- ◊ 600 IU/kg Vit E
- △ 800 IU/kg Vit E
FIGURE 5. a) Multivariate analysis of the effects of different vitamin E treatments in mice infected with *T. cruzi*. Mice (all with 0 ppm selenium) with high positive scores on canonical variate I were characterized by high weights on the day before infection and day 14 (HW (1, 14)), while those with negative scores on canonical variate I were characterized by low weights on the day before infection and day 14 (LW (1, 14)). b) Multivariate analysis of the effects of different levels of vitamin E supplementation in mice infected with *T. cruzi*. Mice with high positive scores on canonical variates analysis (CVA) were characterized by high weights on the day before infection (HW (1)), low weight on day 14 (LW (14)) and high Peakvalue (H Peak), as well as late Firstday (Late Firstday), while those with negative scores on CVA were characterized by low weights on the day before infection (LW (1)), high weights on day 14 (HW (14)) and low Peakvalue as well as early Firstday (Early Firstday).
4 - 2 - 0 - 2 - 0

A • V

200 IU/kg Vit E

400 IU/kg Vit E

600 IU/kg Vit E

800 IU/kg Vit E

LW(1, 14)

HW(1, 14)
Canonical Variate I

HW(1)
LW(14)
H Peak

LW(1)
HW(14)
L Peak

200 IU/kg Vit E
600 IU/kg Vit E
400 IU/kg Vit E
800 IU/kg Vit E

Early Firstday
Late Firstday
FIGURE 6. Effect of selenium plus vitamin E supplementation on weight of *T. cruzi*-infected mice. The average weight of individual *T. cruzi*-infected mice in each selenium treatment group was calculated on days 14, 28 and 42, respectively. All mice were administered 400 IU/kg vitamin E. Data are presented as average weight + standard deviation of weights of five mice in each group.
FIGURE 7. Effect of selenium plus vitamin E supplementation (400 IU/kg) on parasitemia of *T. cruzi*-infected mice. Points represent the mean number of blood-form trypomastigotes per milliliter of blood (five mice per group). Standard deviations are not indicated to preserve the clarity of the figure.
FIGURE 8. Effect of selenium plus vitamin E supplementation (400 IU/kg) on mortality of *T. cruzi*-infected mice. The percent survival for each selenium treatment groups (all with 400 IU/kg vitamin E) is presented for different days post-infection.
FIGURE 9. Plasma levels of parasite-specific antibody on day 35 post-infection in mice infected with *T. cruzi*. All mice were treated with different doses of selenium plus 400 IU/kg vitamin E. Levels of anti-*T. cruzi* antibodies were measured in unpooled plasma collected from individual infected mice and pooled normal mouse plasma. Data are presented as the mean absorbance (at 490 nm) of five plasma samples in each selenium treatment group (all with 400 IU/kg vitamin E). Data obtained from normal mouse plasma are presented as the mean absorbance ± 2 standard deviations of duplicate values.
FIGURE 10. Multivariate analysis of effects of different doses of selenium with 400 IU/kg vitamin E on mice infected with *T. cruzi*. Mice with high positive scores on canonical variates analysis were characterized by high weight on the day before infection, days 14 and 28 as well as low Meanpeak, while those with negative scores were characterized by low weight before infection, days 14 and 28 as well as high Meanpeak.
LW(1, 14, 28)  HW(1, 14, 28)

0 ppm Se with Vit E  8 ppm Se with Vit E
2 ppm Se with Vit E  16 ppm Se with Vit E
4 ppm Se With Vit E
DISCUSSION

The results of the present study verify that antioxidants such as vitamin E and selenium may have a beneficial effect during infectious disease. The combined effect of dietary supplementation with these two micronutrients during experimental Chagas' disease is reported here for the first time.

The results obtained from the first phase of the study demonstrate a significant positive effect of Vit E on mice infected with *T. cruzi* (Fig. 5a). In this analysis, weights on the day before infection and day 14 were contributing factors leading to the significant difference mentioned above. The fact that these factors lost their advantage after day 14 in Vit E deficient mice suggests that Vit E deficient mice lost more weight than Vit E supplemented mice with the progression of *T. cruzi* infection. Because loss of weight is an indication of the severity of disease, this observed increased weight loss indicates that Vit E deficient mice are more susceptible to the pathological consequences of *T. cruzi* infection than Vit E supplemented mice. This conclusion is further supported by the fact that supplementation with 400 IU/kg and 600 IU/kg Vit E also led to decreased parasitemia levels and increased longevity and survival rates. The C3HeB/FeJ mice used in this study are highly susceptible to infection with the Brazil strain of *T. cruzi* and normally die with high parasitemias by day 45 of infection when maintained on standard Purina Rodent Chow. The increased longevity and low parasitemias observed in the 400 IU/kg
and 600 IU/kg Vit E treatment groups suggest that dietary supplementation with Vit E above normal levels (45-75 IU/kg; Bendich, 1990) is beneficial during *T. cruzi* infection.

Vit E, as a lipid-soluble vitamin, is absorbed in the free form in the small intestine of mammals (Budowski and Sklan, 1989). Mammalian transport of newly absorbed Vit E is by substances known as chylomicrons which carry Vit E to the bloodstream and tissues via the lymphatic system (Budowski and Sklan, 1989). In tissue, Vit E is concentrated in membranes, including the mitochondrial, microsomal and plasma membrane (Krishnamurty and Bieri, 1963; Bonnetti and Novello, 1976). Although blood and tissue levels of Vit E were not measured in the present study, mice supplemented with Vit E normally achieve elevated levels of Vit E in the blood and tissues. Thus, the result from detecting effects of dietary supplementation with Vit E as used in the present study should be consistent with that from detecting effects of Vit E in the blood and tissues.

Vit E supplementation has been shown to have a positive effect upon the immune response, while deficiency in Vit E compromises the immune system (Wang and Watson, 1994). Vit E has been found to enhance the activity of T helper cells and to have a positive impact on the antibody response, delayed cutaneous hypersensitivity reaction, phagocytosis, mitogen responsiveness, the reticuloendothelial system, and host resistance in a variety of animal models (Shefty and Schultz, 1979). A significant increase in bactericidal activity in peripheral blood leukocytes has been observed in humans after ingestion of 300 mg of Vit E daily for three weeks (Prasad, 1980). Vit E also has been shown to increase IL-2 production in elderly subjects (Meydani et al., 1986). One of the hallmarks of infection with *T. cruzi* is a profound suppression in the production of IL-2
and in the expression of IL-2R (Majumder and Kierszenbaum, 1996). IL-2 is a pivotal cytokine in the growth and differentiation of T and B cells. Although IL-2 levels were not measured in the present study, it is possible that dietary supplementation with Vit E might allow mice to compensate for the suppression in IL-2 production normally resulting from T. cruzi infection. Based upon the results of earlier studies, it is also possible that Vit E supplementation may lead to enhanced immunoglobulin production and improved T and B cell proliferation during infection (Wang and Watson, 1994). In the present study, only parasite-specific antibody levels were measured. Although differences were observed in antibody titers, there was no statistically significant difference observed in parasite-specific antibody levels between different Vit E treatment groups. Future studies will be required to determine if other parameters of the humoral or cell-mediated immune response are impacted by supplementation with Vit E.

The position of the 600 IU/kg Vit E treatment group in Figure 5b indicates that parasites appeared later in this group than in the 400 IU/kg and 200 IU/kg Vit E treatment groups. It also indicates that the 600 IU/kg Vit E treatment group had the highest weight on day 14 and the lowest peak parasitemia among these four treatment groups. Even though parasites appeared latest in the 800 IU/kg Vit E treatment group, the 800 IU/kg group showed lower weight on day 14 and higher peak parasitemia than the 600 IU/kg treatment group. Thus, the 600 IU/kg Vit E treatment group showed the best overall effect. In the same analysis, the 400 IU/kg treatment group showed the second best overall effect. Mortality data could not be included in the CVA because only one value (i.e., percent survival) is obtained for each treatment group. However, it is a very important
factor when evaluating differences among treatment groups. The 400 IU/kg Vit E treatment group showed the highest survival rate on day 53 of infection among all treatment groups. Therefore, the 400 IU/kg was chosen as the optimal dose of Vit E to be used in the second phase of the study based on mortality data and CVA results.

The results of the present study suggest that the immunostimulatory effect of Vit E is apparent only when Vit E is administered in quantities in excess of normally recommended levels. Mice in the 200 IU/kg Vit E treatment group showed a higher mean parasitemia than mice in the Vit E deficient group (Fig. 2), which suggests that 200 IU/kg Vit E may be not sufficient to impact the levels of circulating parasites in infected mice. The level of Vit E present in standard rodent chow is 49 IU/kg (Purina, 1994). The 800 IU/kg Vit E treatment group showed an overall higher mean parasitemia and an almost 3 fold higher mean parasitemia at its peak than any other group. This result suggests that the 800 IU/kg Vit E dose is above the optimal range of Vit E supplementation in this model system. In short-term studies of Vit E toxicity in mice fed α-tocopherol at dose levels of 5% daily for 2 months, no adverse effects were observed (reviewed by Madhavi and Salunkhe, 1996). In a second study performed by Dysmsza and Park (1975), rats were given 0.0035% and 25, 50, 100, 1000 times this concentration for a period of 13 weeks. Food intake and protein efficiencies were found to be significantly reduced after the first 8 weeks. However, there were no changes in hemoglobin levels, serum cholesterol levels or urinary creatine levels. In a 13-week study in which Fischer 344 rats were administered α-tocopheryl acetate by gavage at levels of 125, 500 or 2000 mg/kg body weight per day, no adverse effects were observed on food intake or body weight (Abdo et al., 1986).
results suggest that even high doses of Vit E are not generally toxic in experimental animals. It is not clear why the 800 IU/kg Vit E treatment led to elevated parasitemias in this study. One possibility may be that such high levels of Vit E were actually benefitting the parasite. Levander et al. (1989) found that mice fed levels of Vit E as low as 100 IU/kg Vit E showed high parasitemias during infection with malarial parasite *Plasmodium yoelii*. *Plasmodium yoelii* is particularly vulnerable to oxidative stress, and the authors suggested that elevated levels of Vit E in the host diet might actually benefit the parasite by allowing it to compensate for ROS production in the host.

The results from the second phase of the study demonstrate the positive effect of combined Se and Vit E supplementation in mice infected with *T. cruzi*. Se and Vit E (400 IU/kg) supplemented mice showed lower mean parasitemias and higher survival rates than mice supplemented with Vit E (400 IU/kg) in the absence of Se. This result suggests that combined Vit E and Se supplementation greatly increases the resistance of mice to infection with *T. cruzi*. A previous study in our laboratory indicated that Se supplementation at levels ranging from 2 ppm to 16 ppm provided in drinking water led to reduced parasitemias and increased survival rates in mice infected with *T. cruzi* (unpublished observation). It was also found that 8 ppm Se provided the best positive effect on mice infected with *T. cruzi*. The results of the present study confirm this beneficial role for Se.

Se can be readily absorbed in the gastrointestinal tract, the respiratory tract and the skin. Under normal conditions, levels of Se are higher in the kidney and liver than in the other major body tissues (WHO, 1987). A plasma selenoprotein is responsible for Se
transport (Motsenbocker and Tapple, 1982). In blood, Se is present as plasma selenium and red blood cell-selenium. Dietary supplementation with Se is correlated with increased levels of Se in the blood and tissues.

Spallholz et al. (1973) investigated the effect of dietary Se supplementation on the immunological responses of mice. Mice on a laboratory chow diet (originally containing 0.5 mg selenium/kg) were supplemented with sodium selenite at 0.7 or 2.8 mg/kg. Supplemented mice showed approximately 7 and 30 fold higher antibody titers, respectively, as compared to mice fed the chow diet alone. Sodium selenite injected into mice intraperitoneally at doses 3-5 µg per animal also increased the primary humoral response to sheep red blood cells, and this increase was greatest when the selenium was given prior to, or simultaneously with, the antigen (Spallholz et al., 1975). The doses of Se needed to enhance the immune response in this study were clearly above the normally recommended levels.

In the present study, mice supplemented with 2 ppm, 4 ppm, 8 ppm and 16 ppm Se (plus 400 IU/kg) showed parasite-specific antibody titers of 1/28160, 1/28160, 1/28160 and 1/14080, respectively. Compared with a titer of 1/3520 exhibited by mice in the Se deficient group, Se supplementation did result in an enhancement of antibody production. However, there was no statistically significant difference between the antibody levels of Se deficient mice and those of Se supplemented mice. Further studies will be required to fully evaluate the impact of Vit E and Se supplementation on humoral responses during infection with T. cruzi.

Mice supplemented with Vit E and Se showed large differences in weight,
parasitemia, survival, and parasite-specific antibody titer as compared to mice supplemented with Vit E only. Mice in 2 groups (2 ppm and 8 ppm Se supplemented groups) even showed a weight gain on day 28 of infection while all other mice exhibited weight loss by day 28 in the first phase of the study. All Se supplemented groups had overall lower parasitemias than the Se deficient group, and all Se supplemented mice showed a survival rate of 65% on day 70 of infection while all Se deficient mice showed a survival rate of 10% on the same day of infection. In addition, the parasite-specific antibody titers of Se supplemented groups (except for the 16 ppm Se supplemented group) are higher than those observed for the Se deficient groups. These results indicate that Vit E and Se have a combined positive effect when administered to mice during infection with *T. cruzi*.

Vit E, as an intracellular antioxidant, prevents oxidative damage to polyunsaturated fatty acids in biological membranes (WHO, 1987). It protects against oxidative stress either by catalyzing the destruction of hydrogen peroxide or catalyzing the decomposition of lipid hydroperoxides, thereby interrupting the free radical peroxidative chain reaction. Se and Vit E show a synergistic effect in depressing lipid peroxide formation *in vivo* and *in vitro* in an NADPH-dependent microsomal lipid peroxidation systems (Madhavi et al., 1996). However, these two micronutrients have different antioxidative effects (Zhu et al., 1992). Vit E prevents lipid peroxidation in biomembranes more effectively than Se; while Se prevents free radical production in the cytoplasm as part of glutathione peroxidase as well as protecting -SH groups present in membrane proteins against oxidation.

The exact mechanism by which Vit E and Se are able to enhance the resistance of
mice to *T. cruzi* infection is not known. *T. cruzi* is an obligate intracellular parasite which must invade host cells in order to replicate. The parasite is capable of invading almost any nucleated cell in the body including cells such as macrophages and neutrophils. Activated macrophages and monocytes produce tumor necrosis factor β (TNF-β) in response to free-radical activity (Greenspan and Aruoma, 1994). TNF-β in turn increases the production of additional ROS from macrophages and neutrophils and acts on T cells to enhance the production of IL-2 and the expression of IL-2R, thereby promoting activation of T-cell-respiratory activity and an increase in intracellular ROS (Greenspan and Aruoma, 1994). Also, activated macrophages can produce nitric oxide (NO), which may negatively impact the DNA synthesis of neighboring cells, especially in lymphocytes and thymocytes (Greenspan and Aruoma, 1994). Large quantities of ROS and NO may lead to severe damage in cells surrounding activated phagocytes (Deshpande et al., 1996). Thus, antioxidants are needed to remove these ROS and NO to protect host cells from oxidative stress. In some disease states that are associated with high levels of oxidative stress, it may be necessary to consume dietary antioxidants at levels greater than normally recommended because a sufficient level of antioxidants must be present to completely rule out the negative effects of ROS or NO.

ROS have been postulated to lead to cardiac injury both in animal models and in humans (reviewed by Paraidathathu et al., 1994). Vit E supplementation has been shown to reduce myocardial oxidative stress (Goldfarb et al., 1996). Thus Vit E supplementation may be beneficial in the prevention of cardiac injury. One of the symptoms associated with the chronic stage of Chagas’ disease is damage to heart muscle cells. In the present study,
mice given Vit E below 800 IU/kg showed increased longevity and reduced parasitemias. It is possible that Vit E supplementation functions, in part, to protect the hearts of infected mice from damage caused directly by parasites and by associated damage resulting from oxidative stress. Future studies will be required to investigate this hypothesis.

The result of canonical variates analysis in the second phase of the study indicates that there is a statistically significant difference among Se treatment groups (Fig. 10). Mouse weights on the day before infection, day 14 and day 28, were major contributing factors in this analysis. Although the 0 ppm Se treatment group showed the highest weight on the day before infection, on day 14, and on day 28, this weight advantage was lost after day 28. This result suggests that the 0 ppm Se treatment group lost more weight than the other treatment groups with the progression of *T. cruzi* infection. The relative weight advantage of the 16 ppm and 8 ppm Se treatment groups increased during infection even though they showed lower weights than the 0 ppm Se treatment group at the beginning of the experiment. Mice in the 8 ppm Se treatment group showed the lowest weight loss of all treatment groups over the entire course of infection (Table 1b). The 16 ppm and 8 ppm Se treatment groups also showed low levels of circulating parasites. Although the 4 ppm Se treatment group also showed low parasitemia levels, mice in this group showed a greater weight loss than mice in the 16 ppm and 8 ppm Se treatment groups. These results indicate that mice in the 16 ppm and 8 ppm Se treatment groups were most resistant to the pathological consequences of the infection. According to the results of CVA, the 16 ppm Se treatment appeared to provide the best overall effect followed by the 8 ppm Se treatment. However, the 8 ppm Se treatment group showed the highest survival rate.
(100% on day 66) among all Se treatment groups. When mortality was considered together with the results of CVA, the combination of 8 ppm Se with 400 IU/kg Vit E was judged to provide the best overall beneficial effect during experimental Chagas' disease. In rats, rabbits and cats the acute oral and intravenous LD$_{50}$ of Se as sodium selenite or selenate was found to be 1.5-3 mg per kilogram of body weight. Se also was found to be toxic at higher doses (reviewed by Madhavi and Salunkhe, 1996). No dose of Se used in the present study was found to be toxic.

In conclusion, the present investigation indicates that Vit E supplementation does have a protective effect during infection with *T. cruzi* and that Vit E in combination with Se is particularly valuable in providing enhanced benefit during experimental Chagas’ disease. The results of this study also suggest that 400 IU/kg Vit E and 8 ppm selenium provide the best protective effect in this model of experimental Chagas’ disease. Future research should be focused on analyzing the histology of surviving mice following antioxidant treatment and determining the mechanisms of protective antioxidant activity during experimental Chagas’ disease.
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