FUNCTIONAL CAPACITY OF THE RESIDUAL LEUKOCYTES FROM ZINC DEFICIENT MICE

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ABSTRACT

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Zinc deficiency drastically compromises cell and antibody mediated immune responses in both humans and animals. This is due, in part, to a significant reduction in numbers of leukocytes. Since numerous zinc dependent enzymes are necessary for cell function, it was important to determine the functional capacity of the residual leukocytes from zinc deficient mice *in vitro* while minimizing zinc repletion.

In response to T-cell stimulants, residual splenic lymphocytes from zinc deficient mice could proliferate, produce interleukin-2, and produce interleukin-2 receptors as well as splenocytes from zinc adequate or restricted mice *in vitro*. In response to antigenic stimulation, antibody production per antibody secreting cell was the same for splenocytes from zinc deficient and zinc adequate mice. Thus, the residual splenic lymphocytes of zinc deficient mice were able to carry out many fundamental immune responses.

In contrast, resident peritoneal macrophages from zinc deficient mice have been reported to associate with and destroy fewer *Trypanosoma cruzi* (*T. cruzi*) parasites than macrophages from adequately fed mice. Modifications of previous assays designated herein made it possible for the first time to directly quantitate H_2O_2 production by resident macrophages using *T. cruzi* as the stimulant. It was found that production of H_2O_2 by macrophages stimulated with *T.*

cruzi trypomastigotes or opsonized trypomastigotes but not amastigotes correlated with T. cruzi-macrophage association. Furthermore, it was shown that arachidonate metabolites may be intermediates in trypomastigote stimulation of H_2O_2 production by resident macrophages.

Upon deprivation of dietary zinc, the total amount of T. cruzi trypomastigote-stimulated H_2O_2 produced/ mg macrophage protein was reduced but H_2O_2 production/ parasite associated with the macrophages was unaltered. Also, the reduced T. cruzi-macrophage association in the zinc deficient group was not due to a decrease in long-chain unsaturated fatty acids, which is known to decrease phagocytosis, or to a decrease in production of leukotrienes, which may decrease trypomastigote-macrophage association. Therefore, some other process(es) critical to association and destruction of T. cruzi must be aberrant in macrophages from zinc deficient mice.

Dedicated to

my husband, James Mills

and

my parents, Norman and Barbara Cook

for their patience, love, understanding, and support

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LIST OF ABBREVIATIONS

(a)	aqueous
BSA	bovine serum albumin
Con A	concanavalin A
cpm	counts per minute
CTLL-2	cytotoxic T-lymphocyte line
DHGL	dihomo-gamma-linolenic acid
DMEM	Dulbecco's modified minimal essential medium
DTH	delayed type hypersensitivity
EDTA	disodium ethylene-diaminotetracetate
FCS	fetal calf serum
(g)	gaseous
HETE	hydroxyeicosatetraenoic acid
HVA	homovanillic acid
IL-2	interleukin-2
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
LTC_4	leukotriene C_4
MLC	mixed lymphocyte culture
NADPH	nicotinamide adenine dinucleotide phosphate
PBS	phosphate buffered saline
PFC	plaque forming cell
PGE_2	prostaglandin E_2

PMA	phorbol 12-myristate 13-acetate
PPD	protein purified derivative
RIA	radioimmunoassay
SEM	standard error of the mean
SOD	superoxide dismutase
SRBC	sheep red blood cells
T. cruzi	*Trypanosoma cruzi*
TD	T-cell dependent antigens
T_H-cell	T-helper cell
TI-1	T-cell independent - class 1
TI-2	T-cell independent - class 2
TNP	trinitrophenyl conjugate
TPP	tetraphenylporphyrin

INTRODUCTION

Nutitional deficiencies compromise the immune system (1). Deficiencies in vitamins (2), protein or amino acids (3), essential fatty acids (4) or trace elements (5) rapidly alter immune function causing an increase in susceptibility to infections (7, 8).

A deficiency of the trace element zinc is a prevalent human nutritional problem throughout the world, including the USA (7, 9, 10, 11). Severe cases of zinc deficiency most often occur in developing nations where poor diets, diseases, and opportunities for infection are prevalent. In both zinc deficient humans and animals, rapid and severe depressions occur in antibody and cell mediated immune responses (12-19). These reductions in immune function may be due to a reduction in cell numbers and/or functional capacity of the cells or subsets of lymphoid cells. There is a drastic reduction (50%) in absolute numbers of lymphocytes in the blood, thymus, and spleen of the mouse, yet, the relative percentage of T- and B-cells (types of lymphocytes) remains unchanged at least in the spleen (12). The total number of macrophages in the blood and spleen is also reduced (50%) (20). These reductions in total cell numbers, at least in part, explain the reduced immune responses of the zinc deficient animal. However, the reduction in immune function may also be due to a decrease in functional capacity of the residual lymphocytes. It is possible that cellular functions are compromised since many enzymes including the RNA polymerases are zinc dependent

(7). In the past, the functional capacity of residual T-cells, B-cells, and macrophages has not been carefully characterized while regulating the presence of the deficient nutrient in vitro in either zinc deficient or other nutritionally deficient animal models. To evaluate the functional capacity of T-cells, we examined proliferation and production of interleukin-2 (IL-2, a hormone-like protein secreted by T-cells) by T-cells from zinc deficient mice in response to Concanavalin A (a mitogen) or allogeneic cells. We also examined the acquisition of IL-2 receptors by these T-cells upon stimulation with allogeneic cells. The functional capacity of residual B-cells was determined by measuring the average amount of IgM and IgG secreted per antibody producing cell during a primary response to sheep red blood cells. In previous in vitro studies, the availability of large amounts of zinc present in fetal calf serum (350 ug Zn/dl) had not been considered as a source of zinc that might facilitate repair or improvement in function of lymphocytes from zinc deficient animals or patients. To greatly reduce exogenous zinc in our studies, we utilized autologous serum from zinc deficient mice (50 ug Zn/dl) or a serum free system. The data in chapter 2 shows that T-cells and B-cells from zinc deficient mice have normal or elevated functional capacities regardless of whether zinc was readily available or limited in the culture system.

In contrast to normal functioning of residual T-cells and B-cells, resident peritoneal macrophages from zinc deficient mice have a reduced functional capacity (21, 22). Macrophages, which are important as one of the first lines of host defense against infections,

are not able to associate with or destroy the parasite Trypanosoma cruzi (T. cruzi) in vitro. Thus, susceptibility to some pathogens is increased in zinc deficient mice. A low zinc diet resulted in increased infections among normally resistant mice to Candida albicans (23), and zinc deficient mice have a reduced ability to combat T. cruzi infections (21, 22, 23). T. cruzi infected zinc deficient mice had a 50 fold increase in blood parasitemias and an increased mortality rate as compared to infected zinc adequate mice or noninfected zinc deficient mice (24). The increased susceptibility to T. cruzi infections is probably, in part, due to the inability of macrophages to associate with or destroy T. cruzi. This is especially interesting since these defects of the macrophage function were reversed by a short period of incubation with zinc at five times physiological concentrations (21, 22). Several other metals at similar concentrations did not restore the ability of zinc deficient macrophages to combat T. cruzi (21, 22).

Since destruction of T. cruzi has been correlated with H_2O_2 production by macrophages (25, 26), we examined the ability of zinc deficient resident macrophages to produce H_2O_2. Usually, H_2O_2 production is measured using artificial stimulants like phorbol myristate acetate (PMA, a chemical) or opsonized zymosan (an antibody coated yeast cell extract) with activated rather than resident macrophages. Activated macrophages are normally used to assay for H_2O_2 because the levels of H_2O_2 produced by resident macrophages were previously too low to detect. Since it was necessary to use resident macrophages from zinc deficient mice to measure H_2O_2 production, the assay for

H_2O_2 production was improved. Modifications to accomplish this goal are presented in Chapter 3. Using this assay, H_2O_2 production by resident macrophages from zinc deficient and zinc adequate mice was measured after stimulation with PMA, opsonized zymosan, or the second messenger in opsonized zymosan-stimulated H_2O_2 production, arachidonate (Chapter 5). To minimize the possibility of restoration of macrophage functions in vitro, all studies on resident macrophages from zinc deficient mice were done using bovine serum albumin (0.4 μg Zn/ g albumin) or a serum free system. Further, since PMA and opsonized zymosan were known to activate H_2O_2 production by different mechanisms, it is possible that T. cruzi used yet another route for stimulation of H_2O_2 production. Therefore, it was also necessary to use T. cruzi as the stimulant when measuring H_2O_2 production by zinc deficient and zinc adequate resident macrophages.

However, stimulation of H_2O_2 production by resident macrophages with T. cruzi posed yet another series of problems. T. cruzi were destroyed by the substrate (phenol red) commonly used to assess H_2O_2 production. The concentration of this substrate could not be reduced since it would then become limiting in the reactions. Therefore, an alternative substrate which did not destroy T. cruzi was found, and the assay for H_2O_2 was again altered and improved for use with resident macrophages with T. cruzi as the stimulant (Chapter 3). This assay represents a valuable new procedure that will enable investigators to evaluate H_2O_2 production by resident macrophages using natural pathogens such as T. cruzi (Chapter 4); heretofore, this was

not possible. T. cruzi stimulated H_2O_2 production by zinc deficient and zinc adequate resident macrophages was measured (Chapter 5).

T. cruzi enters the macrophage by active penetration of the macrophage membrane or by phagocytosis. The mechanism(s) for T. cruzi penetration or phagocytosis is unknown. However, it has been shown that fluidity of the macrophage plasma membrane and phagocytosis by macrophages is depressed by an increase in the plasma membrane in the proportion of fatty acids with shorter chain length and more saturation than arachidonate (27, 28). The percentage of these shorter-chain, more-saturated fatty acids have been shown to be elevated in livers of zinc deficient mice (29-35). Indeed, deficiencies in essential fatty acids and zinc share some similar characteristics such as immune disorders, parakeratosis, and diarrhea (36, 37). Perhaps macrophages from zinc deficient mice have an altered fatty acid composition which would reduce membrane fluidity and phagocytosis and therefore decrease the association of T. cruzi. Fatty acids such as arachidonic, linolenic, linoleic, and stearic acid added exogenously have also been shown to stimulate production of oxygen metabolites by macrophages, and as chain length decreased or saturation increased these fatty acids were less stimulatory for production of the oxygen metabolites (38, 39). The mechanism for T. cruzi stimulated H_2O_2 production is unknown. However, T. cruzi does stimulate the release of leukotrienes (40) which are lipoxygenase metabolites of the fatty acid, arachidonate. In addition, fatty acids are intermediates in stimulation of H_2O_2 production by opsonized zymosan (41) and may also be involved in T. cruzi stimulated

H_2O_2 production. Alterations in fatty acid composition of zinc deficient macrophages could cause a reduction in both the number of T. cruzi associated with the macrophage and the degree of stimulation of H_2O_2 production. Therefore, the fatty acid composition of phospholipids from zinc deficient and zinc adequate resident macrophages was determined (Chapter 5).

In addition, there is the possibility that release of fatty acids upon stimulation of the macrophage by T. cruzi may be a zinc dependent process. One process for liberation of fatty acids from phospholipids is known to be zinc dependent. Phospholipase C, which in conjunction with diacylglycerol lipase liberates fatty acids from phospholipids, requires zinc for activity (42). Perhaps, in the macrophages from zinc deficient mice, phospholipase C lacks adequate amounts of zinc and thus has reduced activity. To examine this possibility, T. cruzi stimulated release of radiolabelled arachidonic acid and it's metabolites from zinc deficient and zinc adequate macrophages was measured while carefully regulating zinc levels in vitro (Chapter 5).

In summary, studies were performed to determine the ability of T-cells from zinc deficient mice to proliferate and produce interleukin-2 and interleukin-2 receptors, the ability of B-cells from deficient mice to produce antibodies, and the ability of zinc deficient macrophages to produce H_2O_2 and release fatty acids. Macrophage fatty acid composition was also examined. In contrast to previous studies, the level of zinc introduced in vitro was carefully regulated. Thus, real and not artifactual functional capacities of leukocytes from zinc deficient and zinc adequate mice were studied.

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Chapter 1

LITERATURE REVIEW

The purpose of this research was to analyze the functional capacity of leukocytes from zinc deficient mice. T-cell proliferation was analyzed as well as two processes important in stimulating the proliferation, IL-2 production and IL-2 receptor production by T cells. The ability of B-cells to produce antibodies was measured on a per cell basis. Macrophages, which are important as one of the first lines of defense against pathogens, was also analyzed. Measurements of macrophage production of toxic oxygen metabolites, release of fatty acids, and macrophage fatty acid composition were performed. Therefore, this review will provide the reader with information concerning zinc deficiency and it's effects on the immune system. Also, the possible roles for zinc in the phagocytosis and destruction of pathogens by macrophages will be discussed.

I. Zinc Deficiency

A.) Causes of Zinc Deficiency. A suboptimal intake of zinc in the human diet is not uncommon (1, 2, 3, 4). Severe zinc deficiencies are most prevalent in underdeveloped nations since people from these countries consume large quantities of unleavened grain products containing phytate, which chelates zinc making it unavailable for absorption from the intestine (1). Deficiencies in zinc are not confined to the underdeveloped nations; in fact, it has also been observed in middle and upper income families of the United States (2, 3). These deficiencies were probably caused by improper eating

habits resulting in imbalanced diets. In addition, a study of children from low income families in the Denver area revealed that several children were zinc deficient; they had poor taste acuity, slow growth and low hair zinc levels (2). Upon supplementation with zinc, these conditions were alleviated (5). It should be noted that children and pregnant women (6) who may have an increased nutrient requirement for zinc are at a greater risk of becoming deficient.

A genetic disorder called Acrodermatitis enteropathica also results in a deficiency in zinc (1). Acrodermatitis enteropathica is an autosomal recessively inherited trait which reduces the individuals capacity to absorb zinc from the diet. These patients have a severe dermatitis, reduced plasma zinc levels, reduced cellular immunity, and increased susceptibility to infections (7, 8), common characteristics accompaning zinc deficiency. Upon zinc therapy, these symptoms are alleviated (7, 8). A similar disorder occurs in Freisian cattle (9). It has been suggested that the reduced absorption of zinc is due to lack of a zinc binding protein in the intestine of patients with Acrodermatitis (10).

In addition to improper diets or malabsorbtion of zinc, several disease states may result in zinc deficiency. With catabolism of body mass, there will be loss of zinc as well as a high zinc requirement for repair. Illnesses resulting in zinc deficiency include chronic renal disease, burns or psoriasis, malignancies, parasitic infections, diabetes, cirrhosis of the liver, alcoholism, and collagen disease (11). Drug treatments can also cause depletion of zinc; these include antimetabolites, antianabolic agents, penacillamine

therapy, prolonged intraveneous therapy with nutrient solutions containing insufficient amounts of zinc, and oral contraceptives (11).

B.) Alterations in the immune system by zinc deprivation.

Human studies show that zinc deficiency alters T-cell and B-cell responses. A female child with Acrodermatitis enteropathica was found to have many alterations in her immune system (12). Analysis of her peripheral lymphocytes showed that there was a decrease in the number of B- and T-cells. In addition, the lymphocytic response per 10^6 cells to mitogens specific for T-cells or B-cells (Con A, PHA, PWM) was reduced. Delayed-type-hypersensitivity responses were also reduced. She also had hypogammaglobulemia, a characteristic of Acrodermatitis. Upon zinc therapy, all responses returned to normal levels.

Animal studies have also shown that cellular and antibody mediated responses are affected by zinc deprivation in vivo. The T_H-cell dependent antibody mediated response per spleen to sheep red blood cells (SRBC) is 10% of controls (13), but upon reconstitution of the zinc deficient mice with thymocytes, the response increases to 61% of controls (13). Therefore, the response may be due to reduced numbers and/or impairment of B-cells and/or T-cells. The reduced antibody mediated response to SRBC is partly the result of reduced numbers of splenocytes since the response per 10^6 splenocytes is normal (14). However, the ability of the residual B-cells to produce antibodies was unknown. Chapter 2 examines the average

amount of antibody production per antibody producing cell from zinc deficient and zinc sufficient mice. There is also loss of some T-cell functions including cytotoxic T-cell responses against allogeneic tumor cells and decreased delayed-type-hypersensitivity (DTH) responses (inflammatory responses mediated by T_D-cells and macrophages) to dinitrofluorobenzene (15, 16).

The altered T-cell mediated responses seen with zinc deprivation may be due to impairment of normal development of T-cells in the thymus or to impairment of the functions of more mature T-cells. The functional capacity of the residual T-cells had not been examined. Chapter 2 discusses the ability of T-cells to proliferate and produce interleukin-2 and interleukin-2 receptors. T-cell development seems to be altered. Nash et. al. (17) found an increase in number of immature T-cells in spleens of zinc deficient mice as determined by autologous rosette formation. Impaired development of T-cells may be a result of alterations in the thymus. In the zinc deficient animal the thymus is severely atrophied with preferential loss of the thymic cortex, the location of immature thymocytes (18). The thymic atrophy is caused, in part, by the high corticosterone levels found in the zinc deficient animals (19). Adrenalectomized zinc deficient mice are protected from thymic atrophy (20); yet, there is only marginal (20%) improvement in antibody mediated responses per spleen to SRBC, a T-cell dependent antigen. In addition, with increased severity of the zinc deficient condition, in both the adrenalectomized and nonadrenalectomized mice, the T-cell dependent response to SRBC continues to decrease on a per spleen basis (21). Therefore,

thymic atrophy due to high corticosterone levels is not the only factor contributing to impairment of the response to SRBC by zinc deficiency. Not only is the thymus atrophied but several thymic hormones are altered with zinc deficiency. These hormones may play a role in development of T-cells in the thymus (22, 23). Iwata (22) and Chandra (23) observed that zinc deficient mice had reduced activity of thymulin, a zinc dependent hormone (24). Another thymic hormone, thymopoietin was found to be present in reduced levels in the serum of zinc deficient patients (25). A reduction in these hormones may cause improper differentiation and maturation of T-cells in the thymus and peripheral lymphoid organs of zinc deficient individuals. Improper development of the T-cell could at least in part explain altered T-cell responses upon zinc deprivation.

B-cell development may also by halted at an intermediate stage; that is, the development of the immature B-cell in the bone marrow to the mature peripheral resting B-cell may be impaired. Ability to respond to certain polyclonal B-cell mitogens and/or antigens arise at different stages during ontogeny. Mitogens which stimulate B-cells include dextran sulfate, lipopolysaccharide (LPS) and protein purified derivative (PPD). Dextran sulfate stimulates immature B-cells (26-28). LPS stimulates B-cells in intermediate stages of development, while PPD stimulates more mature resting B-cells (26-29). It has been demonstrated that when equal numbers of lymphocytes from zinc deficient mice are stimulated with dextran sulfate or LPS, the response is twice that of controls (14, 30). However, when cells are stimulated with PPD there is not a significant difference from the

controls (14). T-cell independent antigenic responses by immature B-cells were also elevated. Responsiveness to T-cell independent (TI) antigens develops at different stages of B-cell ontogeny. Trinitrophenyl conjugated LPS (TNP-LPS), a "TI-1" antigen, stimulates immature B-cells; whereas, TNP-Ficoll, a "TI-2" antigen, stimulates B-cells in intermediate stages of development (31). Again, the in vitro mitogenic response of splenocytes of zinc deficient mice to either of these TI-antigens is elevated (14, 30). The increased B-cell responses to antigens or mitogens which stimulate relatively immature B-cells suggests that resting B-cells from zinc deficient mice might be halted in some intermediate stage of development.

Although B-cell and T-cell subsets appear to be differentially affected by zinc deprivation (14), the relative percentage of B- and T-cells in the spleen does not change in zinc deficient versus zinc adequate mice (32). However, the total number of lymphocytes in the spleens of zinc deficient mice is reduced (14) (50% of controls). The total number of mononuclear phagocytes, phagocytic cells of the immune system, is also reduced (50%) in the spleens of zinc deficient mice (33). Taken together, the reduced cell and antibody mediated responses are in part due to reduced cell number. An additional explanation could be reduction in the ability of the residual leukocytes to carry out specific functions especially since about 200 zinc metalloenzymes have been described (1). The zinc dependency of these enzymes has usually been identified by loss of enzyme activity due to removal of zinc by chelation (34). Amoung the zinc dependent enzymes are thymidine kinase, DNA dependent RNA polymerase, and

terminal deoxynucleotidal transferase. Loss of enzyme activity due to loss of zinc could explain several alterations in cellular functions of zinc deficient animals. However, in a zinc deficient state, loss of enzyme activity due to loss of zinc has not been demonstrated. In fact, Luecke et. al. has shown that in the zinc deficient weanling pig the activity per cell of several zinc dependent enzymes remains normal; yet, the total amount of the enzyme in the tissue is usually reduced due to atrophy of the organ (35). If there is altered enzyme activity, one would expect to find altered functional capacities. Therefore, to determine if zinc regulates the functional capacity of B-cells and T-cells, proliferation and cytokine production by residual lymphocytes from zinc deficient mice was measured in Chapter 2.

It has been shown that some functional capacities of peritoneal macrophages from zinc deficient mice are aberrant. This will be discussed in detail in section I C. Although the total number of peritoneal macrophages from deficient mice is normal (33) and they are able to phagocytose normal numbers of latex beads (33), they have a reduced capacity to ingest and destroy pathogens (36, 37). The inability of the zinc deficient macrophages to destroy T. cruzi was further studied in Chapter 5.

In summary, zinc deficiency alters lymphocytic responses in both animals and humans. B-cells may be arrested in intermediate stages of maturation. T-cell mediated responses are decreased although thymic atrophy was of only minor importance in T-cell impairment. However, the reduced levels of thymic hormones in zinc deficient

individuals may also arrest development of T-cells. Macrophages are unable to associate and destroy the pathogen T. cruzi; however, they are able to associate with inert particles. The reduced immune responses are in part due to reduced cell number and may also be due to reduced functional capacity of the residual leukocytes.

C.) Resistance to infection. Zinc deficient mice are more susceptible to pathogens than their zinc adequate counterparts. Salvin and Robin (38) demonstrated that a low zinc diet increased the infection of normally resistant mice to Candida albicans. Also, Fraker et. al. (39) showed that zinc deficient mice have a reduced ability to combat Trypanosoma cruzi, an obligate intracellular parasite which causes Chagas' disease. This was demonstrated by a 50 fold increase in blood parasitemias in zinc deficient mice compared to controls (39). In addition, zinc deficiency and infection with T. cruzi had a synergistic effect on the death rate (39). Twenty-two days after infection, 80% of the infected zinc deficient mice died, whereas there were no deaths among the infected zinc adequate mice or the uninfected zinc deficient mice (39).

The inability of the zinc deficient mice to protect against infection by T. cruzi is most likely due, in part, to the inability of the resident macrophages from the zinc deficient mice to phagocytose and destroy the parasite. In vitro the percentage of macrophages associated (a term including membrane bound and phagocytosed parasites) with T. cruzi and the number of T. cruzi per 100 macrophages

was substantially reduced in the zinc deficient groups at time zero after infection (36, 37). After 24 hours, macrophages from the zinc deficient groups were not able to destroy as many parasites as macrophages from the zinc adequate and the pair-fed zinc adequate groups (36, 37). That is, at 24 hours as compared to zero hours, there was a reduction (20-50%) in the percentage of macrophages associated with T. cruzi and the number of T. cruzi per 100 macrophages in the zinc adequate groups but not the zinc deficient groups.

This defect was particularly interesting because it was readily reversed by in vitro addition of zinc. Before infection, a 1 hour treatment of the macrophages with 10μg $ZnCl_2$/ml (5 times physiological serum concentrations of zinc) repaired the ability of the zinc deficient groups to associate with and destroy the T. cruzi (36, 37). The pretreatment with zinc did not affect the ability of the macrophages from zinc adequate mice to associate with and destroy the T. cruzi (36, 37). This ability of $ZnCl_2$ to reverse the defects of zinc deficient macrophages was specific in that pretreatment in vitro with several other trace metals ($CuCl_2$ and $NiSO_4$) at five times physiological concentrations did not significantly reverse the defect of the zinc deficient macrophages to associate with and destroy the parasite (36, 37). Pretreatment with $MnCl_2$ had a slight restorative affect on the ability of the zinc deficient macrophages to associate with the T. cruzi (36, 37). In summary, zinc may play some role in the association with and destruction of T. cruzi by resident macrophages. Thus, zinc deficient macrophages provided an excellent

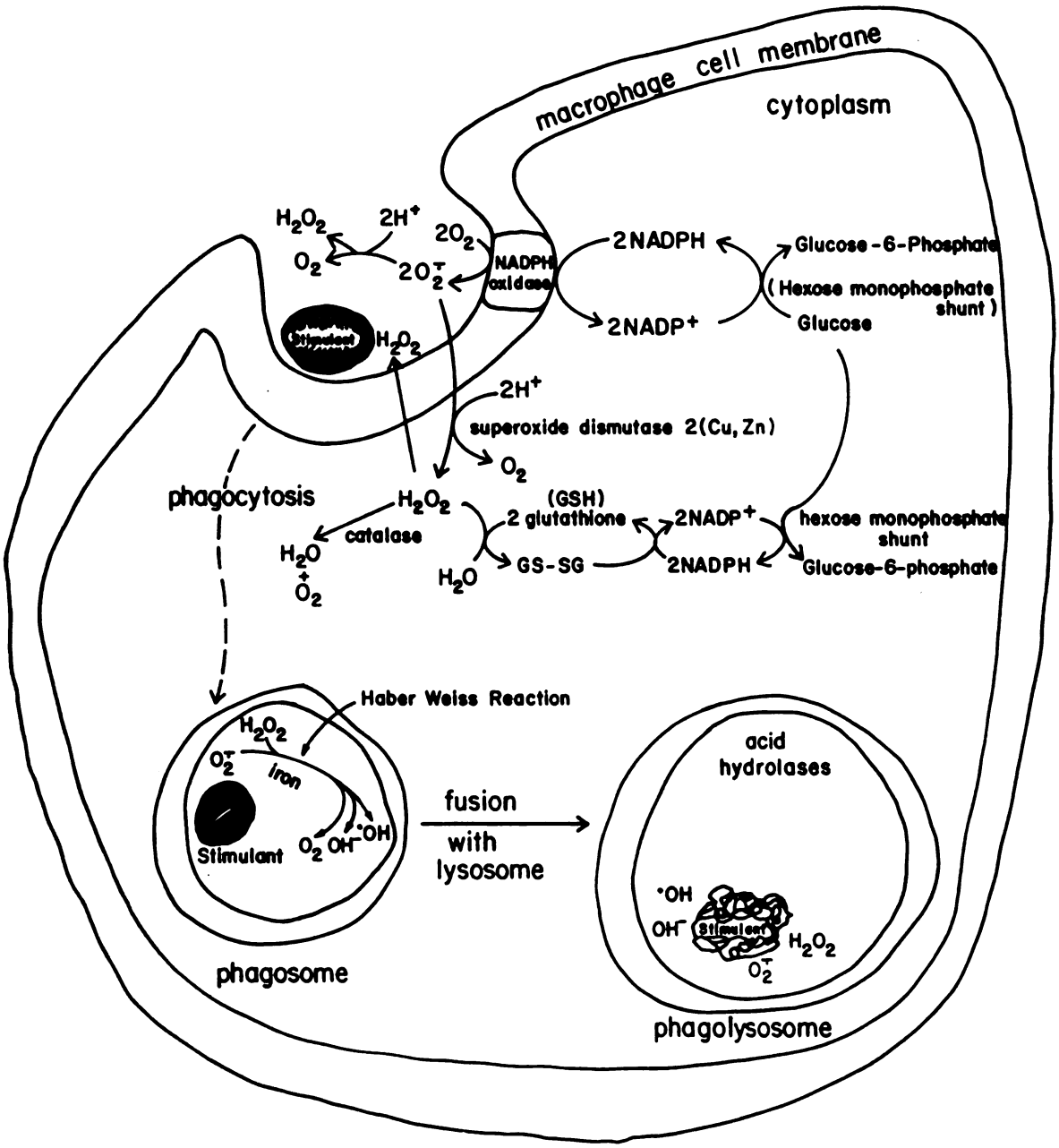
opportunity to investigate possible zinc dependent mechanisms in the association with and destruction of T. cruzi (see chapter 5).

II. Possible Roles for Zinc in the Destruction of Pathogens by Macrophages.

Since macrophages from zinc deficient mice are unable to destroy the pathogen T. cruzi (36, 37) and T. cruzi are destroyed by toxic oxygen metabolites produced by macrophages (40), perhaps zinc participates in the "oxygen burst", the consumption of oxygen and production of toxic oxygen metabolites by leukocytes. Other metals such as iron and copper (41) are known to participate in the "oxygen burst". However, heretofore, the importance of zinc in the "oxygen burst" has gone unrecognized. In this section, possible roles for zinc in the production of toxic oxygen metabolites and the impaired destruction of pathogens by macrophages from zinc deficient animals will be discussed. Also, since macrophages from zinc deficient mice have reduced ability to associate with T. cruzi, there will be a discussion on possible roles for zinc in macrophage-T. cruzi association including zinc-membrane interactions and zinc in fatty acid production for membrane phospholipids.

A.) Production of Toxic Oxygen Metabolites by Mononuclear Phagocytes: Possible Roles for Zinc. The "oxygen burst" (Figure 1) can be activated by phorbol myristate acetate (PMA) (42, 43), a small molecule which can diffuse into the cell. The "burst" can also be

Figure 1. The "oxygen burst" - Mechanisms for production of oxygen metabolites by macrophages.


macrophage cell membrane
cytoplasm
H_2O_2
$2H^+$
$2O_2$
O_2
$2O_2^-$
NADPH oxidase
2NADPH
2NADP+
Glucose-6-Phosphate
(Hexose monophosphate shunt)
Glucose
Stimulant
H_2O_2
$2H^+$
superoxide dismutase 2(Cu, Zn)
O_2
phagocytosis
H_2O_2
catalase
H_2O
O_2
(GSH)
2 glutathione
H_2O
GS-SG
2NADP+
2NADPH
hexose monophosphate shunt
Glucose-6-phosphate
Haber Weiss Reaction
H_2O_2
O_2^-
iron
Stimulant
O_2 OH^- $^{\cdot}OH$
fusion
with
lysosome
phagosome
acid
hydrolases
$^{\cdot}OH$
OH^-
Stimulant
H_2O_2
O_2^-
phagolysosome

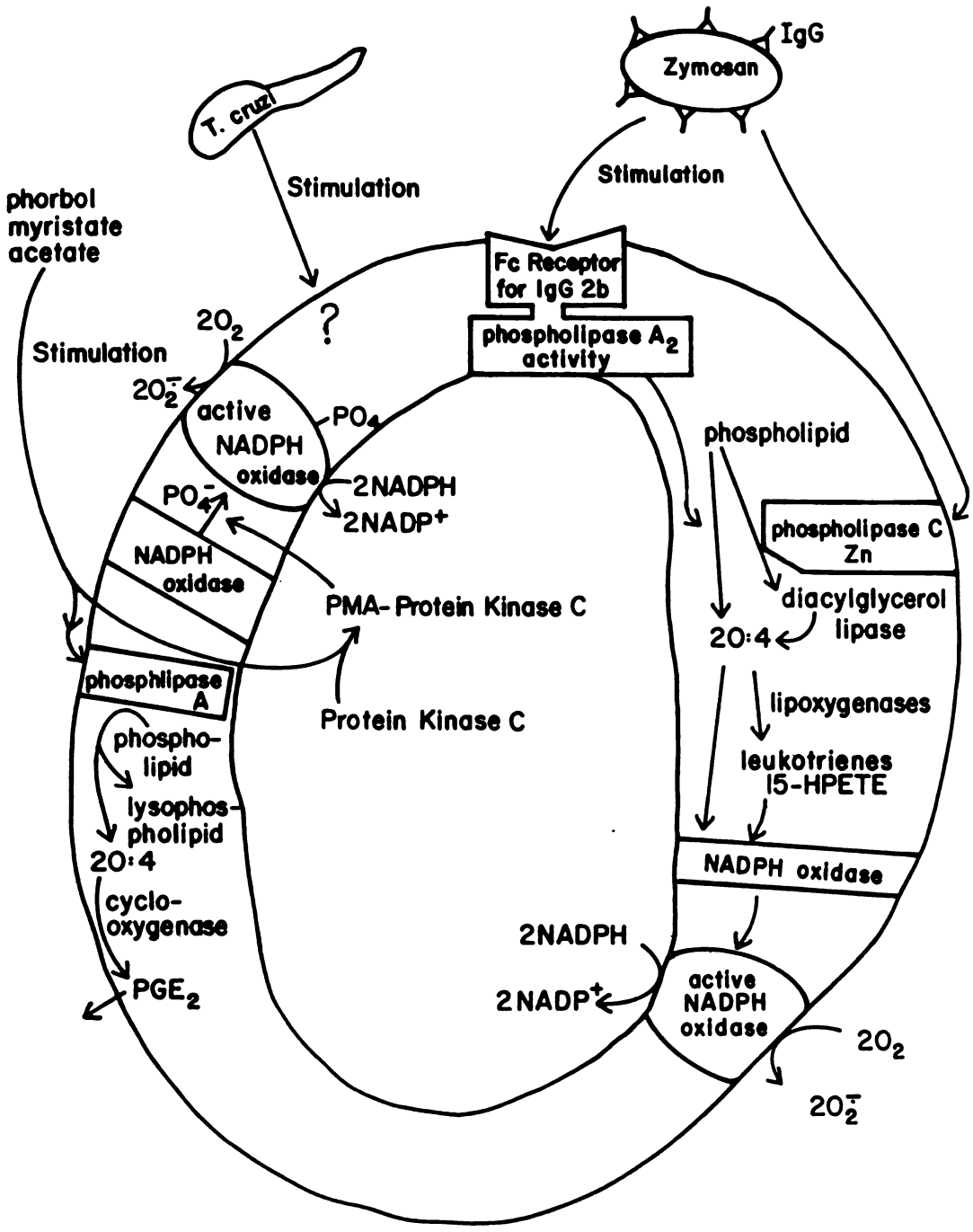
activated by stimulants which are phagocytosed via specific receptors such as opsonized zymosan (42), an antibody coated yeast cell extract, or T. cruzi (44, chapter 3 and 4), a pathogen. These agents eventually promote activation of NADPH oxidase, an enzyme located in the plasma membrane, which, in turn, catalyzes the production of superoxide (O_2^-) from molecular oxygen in the extracellular environment or phagosome/phagolysosome. This reaction requires two NADPH molecules which are provided by the hexose monophosphate shunt in the cytoplasm. The O_2^-, then, either nonenzmatically dismutates to H_2O_2 or superoxide dismutase in the cytoplasm catalyzes the dismutation. As O_2^- and H_2O_2 are produced, they can diffuse through the plasma membrane. Extracellular/phagosome/phagolysosome O_2^- and H_2O_2 are used to destroy pathogens while cytoplasmic O_2^- and H_2O_2 are scavenged by superoxide dismutase and catalase, respectively, to protect the macrophage. Glutathione peroxidase also scavenges H_2O_2 while oxidizing glutathione. Within the phagosome/phagolysosome, H_2O_2 reacts with O_2^- to produce O_2, OH^- and $\cdot OH$. The O_2^-, H_2O_2, and $\cdot OH$ are toxic to various pathogens.

Phagolysosomes also contain lysosomal enzymes. Myeloperoxidase which catalzyes the production of highly reactive hypohalous acids from H_2O_2 is present in phagolysosomes of monocytes, immature mononuclear phagocytes of the peripheral blood, and polymorphonuclear leukocytes but not macrophages (45). Therefore, the mature macrophage is unable to produce hypohalous acids. Thus, we will not be concerned with the possible effects of zinc deficiency on these

processes since all experiments reported herein were done with mature macrophages.

Mechanisms of the "oxygen burst" will now be described while focussing on possible roles for zinc in these processes. The mechanisms of stimulation of NADPH oxidase by PMA and opsonized zymosan are known (Figure 2). PMA stimulates NADPH oxidase by binding to protein kinase C (46, 47) which, in turn, phosphorylates and thus activates NADPH oxidase (48-53). On the other hand, opsonized zymosan stimulates NADPH oxidase via arachidonic acid released from phospholipids (20:4) (48, 49, 54, 55, 56, 57). The following is the sequence of events most likely to occur upon stimulation of macrophages by opsonized zymosan. The opsonized zymosan binds to receptors on macrophages for the Fc portion of the antibody. Suzuki et. al. (58) showed that a Fcγ2b binding protein of macrophage membranes has phospholipase A_2 activity since it specifically cleaves fatty acids from the C2 position of the glycerol backbone of phosphatidylcholine. The activity of this protein increased four fold upon binding of IgG2b (58). Therefore, upon binding of opsonized zymosan to Fcγ2b receptors, the phospholipase A_2 portion of the receptor could liberate 20:4 from phospholipids in the macrophage plasma membrane. A considerable amount of the fatty acid (25%) in macrophage phospholipids is 20:4 (59). Once 20:4 is liberated from the plasma membrane phospholipid, it may then stimulate NADPH oxidase. It has been shown that 20:4 and lipoxygenase metabolites of 20:4 such as leukotrienes, 15-HETE or 15-HPETE, stimulate NADPH oxidase and that this stimulation is not inhibited by cyclo-oxygenase or lipoxygenase inhibitors

Figure 2. The "oxygen burst" - Mechanisms for activation of NADPH oxidase.


IgG
Zymosan
T. cruzi
Stimulation
Stimulation
phorbol
myristate
acetate
Fc Receptor
for IgG 2b
phospholipase A2
activity
?
20₂
Stimulation
20₂⁻
active
NADPH
oxidase
PO4
2NADPH
2NADP+
PO4⁻
NADPH
oxidase
PMA- Protein Kinase C
phosphlipase
A
Protein Kinase C
phospho-
lipid
lysophos-
pholipid
20:4
cyclo-
oxygenase
PGE2
phospholipid
phospholipase C
Zn
diacylglycerol
lipase
20:4
lipoxygenases
leukotrienes
15-HPETE
NADPH oxidase
2NADPH
2NADP+
active
NADPH
oxidase
20₂
20₂⁻

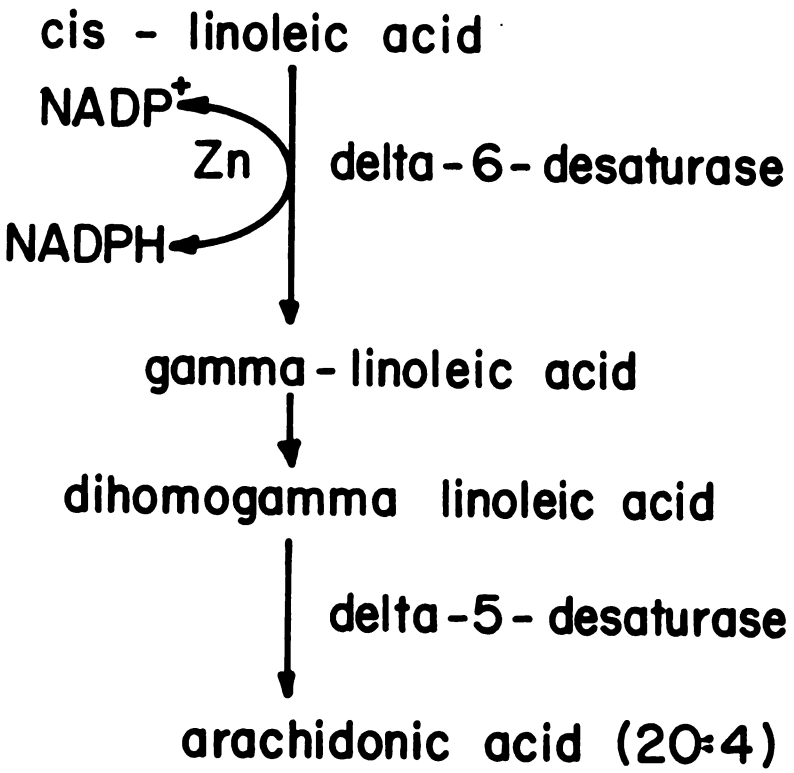
(48, 54, 55, 56, 57, 60). In contrast, 20:4 metabolites of the cyclo-oxygenase pathway, the prostaglandins, are not stimulatory for NADPH oxidase (48, 54). Thus, upon binding of opsonized zymosan to Fc&2b receptors, phospholipase A_2 activity of the receptor liberates 20:4 from membrane phospholipids and 20:4 or a lipoxygenase (but not a cyclo-oxygenase) metabolite of 20:4 stimulates NADPH oxidase for the production of superoxide.

Resident peritoneal macrophages have three phospholipase activities (61-65): a phospholipase A_2 active at pH 4.5, a Ca^{2+}-dependent phospholipase A_2 active at pH 8.5, and a phosphatidylinositol-specific phospholipase C. The particular phospholipases involved in the degredation of phospholipids differs with different stimulants. Zymosan stimulates degradation via both phospholipase A and phospholipase C whereas PMA stimulates deacylation of phosphatidylinositol via phospholipase A (64). The PMA stimulated release of 20:4 does not activate NADPH oxidase (Figure 2) since, with this stimulant, the 20:4 is converted to prostaglandins (66, 67) which are not stimulatory for NADPH oxidase (48, 54). 20:4 released by phospholipase A and the lipoxygenase metabolites are stimulatory for the "oxygen burst" (48, 54, 55, 56, 57). Further evidence for the involvement of phospholipase C in the stimulation of the "oxygen burst" includes an increase in O_2 uptake and hexose monophosphate shunt activity and a 41% decrease in phospholipid content as determined by lipid-bound phosphate content upon addition of phospholipase C to polymorphonuclear leukocytes (68). Phospholipase C stimulation of

the "oxygen burst" probably involves diacylglycerol lipase catalyzed release of 20:4.

There are several possible roles for zinc in the stimulation of NADPH oxidase by 20:4. Zinc may be important for the release of 20:4 from phospholipids, since phospholipase C, a zinc dependent enzyme (69, 70), in conjunction with diacylglycerol lipase can liberate 20:4 from phospholipids (Figure 2) (70). Phospholipase C has two zinc atoms in the active site (70). Both of which are required for activity (70). Removal of even one zinc atom reduces the enzymatic cleavage of phosphatidylcholine to 3-11% of control and removal of the second zinc atom reduces the activity to less than 1% (70). These zinc atoms are tightly bound to the active site (70). In fact, EDTA does not seem to remove zinc from the enzyme since addition of EDTA has no effect on enzyme activity (70). Zinc has also been implicated in the regulation of phospholipase A_2 activity (71-73). Phospholipase A_2 liberates fatty acids from the C2 position of the phospholipid glycerol backbone. Manku et. al. (72) have suggested that in the presence of physiological levels of zinc, dihomo-gamma-linolenic acid (DHGL) is mobilized from plasma membrane phospholipids of the rat superior mesenteric vascular bed. DHGL is the immediate precursor to either 20:4 (Figure 3) or prostaglandins of the 1 series. If DHGL is converted to 20:4, mobilization of DHGL could play a significant role in stimulating the oxygen burst via 20:4. Zinc mobilization of DHGL for prostaglandin synthesis would not be stimulatory for NADPH oxidase since prostaglandins do not stimulate NADPH oxidase. However, inhibition of prostaglandin

Figure 3. Synthesis of arachidonate from cis-linoleic acid.


cis - linoleic acid
NADP+
Zn
NADPH
delta-6-desaturase
gamma-linoleic acid
dihomogamma linoleic acid
delta-5-desaturase
arachidonic acid (20:4)

synthesis may allow DHGL or its metabolite, 20:4, to stimulate NADPH oxidase. This is a possibility since addition of 2mM zinc in vitro has been shown to inhibit prostaglandin synthesis by polymorphonuclear leukocytes (73). In addition, perhaps DHGL itself could stimulate NADPH oxidase since shorter chain fatty acids can, albeit to a lesser extent, stimulate NADPH oxidase (48, 54). In contrast to the phospholipase A_2 activity in the presence of zinc reported by Manku et. al. (72), Wells (74) has reported that zinc inhibits the calcium dependent activity of phospholipase A_2 from Crotalus adamanteus (snake) venom by binding to the active site and inducing conformational changes in the enzyme. Inhibition of phospholipase A_2 activity by addition of zinc has also been reported by others (75, 76). In summary, zinc may be important in the release of 20:4 from phospholipids via phospholipase C - diacylglycerol activity or some phospholipase A_2 like activity.

Zinc may also be important in the stabilization of 20:4 against oxidation and enhance the probability of stimulation of NADPH oxidase by 20:4. It is known that zinc or iron can complex with 20:4 and oxygen (77, 78). Iron catalyzes the oxidation of 20:4 whereas zinc does not (78).

$$20{:}4 + O_2 + Fe^{2+} \longrightarrow [20{:}4, O_2, Fe^{2+}]_{complex} \longrightarrow \text{lipid peroxides} + O_2^- + Fe^{3+}$$

$$20{:}4 + O_2 + Zn^{2+} \dashrightarrow [20{:}4, O_2, Zn_2^{+}]_{complex}$$

Zinc also competes with iron for formation of this complex (78). Perhaps, zinc stabilizes the 20:4 so that 20:4 is not available for

oxidation in the iron complex and therefore can stimulate the NADPH oxidase.

Zinc may also play a role in the catalysis of O_2^- production by NADPH oxidase since nucleotides, such as NADPH, are able to complex with zinc as determined by elution patterns of NADPH from Sephadex G-10 columns equilibrated with zinc ions (79, 80). Zinc binds to NADPH in a 2:1 molar ratio (80). The first zinc atom binds between the monophosphate on the C2 of the adenine-ribosyl portion and the diphosphate group (80). The second zinc atom binds to the remaining oxygen of the monophosphate and the diphosphate, thus forming a ten member ring (80). When zinc complexes with NADPH, it interferes with oxidation of NADPH by making it unavailable as an enzyme substrate (80). The activity of the enzymes, NADPH oxidase derived from liver microsomes and mixed function oxidases from smooth endoplasmic reticulum, are inhibited in the presence of zinc (K_i =7.22 μM Zn^{2+}) (80). In the presence of physiological or deficient levels of zinc, the formation of the Zn_2-NADPH complex probably plays only a minor, if any, regulatory role in the oxygen burst by macrophages since, with zinc deficiency, H_2O_2 production by macrophages is decreased (Chapter 5); thus, it is doubtful that NADPH oxidase activity would be increased. However, supraphysiological levels of zinc may inhibit NADPH oxidase via complex formation with NADPH. Perhaps this explains the inhibition of oxygen consumption and hexose monophosphate shunt activity in polymorphonuclear leukocytes and peritoneal macrophages by high concentrations of zinc <u>in vitro</u> (81-84).

Superoxide, produced by NADPH oxidase, either nonenzymatically dismutates to H_2O_2, or superoxide dismutase (SOD) catalyzes the dismutation (Figure 1). The enzymatic dismutation of O_2^- is metal dependent. There is a cytoplasmic Cu/Zn SOD and a mitochondrial Mn-SOD. SOD is a dimer of two identical subunits (85). Each subunit of the cytoplasmic SOD contains one molecule of copper and one molecule of zinc located far apart (about 34 angstroms) (85). The two copper ions are catalytic cofactors whereas the two zinc ions are structual cofactors (86). Upon removal of zinc from Cu/Zn SOD, the enzyme activity at pH6 is still 90% of the intact Cu/Zn SOD (86). At pH>7, the copper ion migrates to the zinc free site (86). So, at pH8.5, the zinc free SOD has only 50% activity (86). The rate constants for interaction of O_2^- with zinc free SOD at pH6 is similar to the intact Cu/Zn SOD (114). From this, the authors suggested that the catalytic mechanism (a ping-pong mechanism) for zinc free SOD is the same as the intact Cu/Zn SOD. Zinc is thought to play a structural role since it has been shown that zinc stabilizes SOD against inactivation by SCN^- (86). The major biological role for Cu/Zn SOD has not been elucidated. It may be important in the production of H_2O_2 for destruction of pathogens or it's major role may be scavenging O_2^- to protect the macrophage from O_2^- that has diffused through the membrane. Whatever the biological role for SOD in the macrophage, zinc is not crucial for activity of the Cu/Zn SOD.

The nonenzymatic dismutation of O_2^- may also be metal dependent. Iron has been shown to react with O_2^-. Furthermore indirect evidence suggests that zinc may also be involved in some reactions

with oxygen species. The reaction with iron is called the Haber-Weiss reaction (Fig. 2) (41).

$$Fe^{3+} + O_2^- \longrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + \cdot OH$$

$$\cdot OH + H_2O_2 \longrightarrow H_2O + HO_2\cdot$$

$$HO_2\cdot + H_2O_2 \longrightarrow O_2 + H_2O + \cdot OH$$

$$\cdot OH + Fe^{2+} \longrightarrow Fe^{3+} + OH^-$$

The resulting oxygen metabolites are OH^- and the toxic $\cdot OH$. These reactions occur at acidic pH's found in the phagosome/phagolysosome. The phagosome/phagolysosome contains the engulfed pathogen/stimulant (Figure 1). These reactions do not occur at higher pH's found in the cytoplasm. This is important since production of highly reactive oxygen metabolites in areas other than the phagolysosome could destroy the leukocyte.

Zinc may also be involved in the nonenzymatic dismutation of O_2^-. There is evidence, albiet inconclusive, that zinc catalyzes reactions with oxygen. In the following reactions, reduction of oxygen in the presence of zinc and bipyridine (bipy) yields highly reactive radicals and zinc peroxides (87).

$$Zn^{II}(bipy)_2^{2+} + O_2 + e^- \xrightarrow{-0.5V} [Zn^{II}(bipy)_2OO\cdot]^+$$

$$[Zn^{II}(bipy)_2OO\cdot]^+ + e^- \longrightarrow Zn^{II}(bipy)_2(O_2)$$

These chemical reactions were determined electrochemically under aprotic conditions (dimethylformamide). Aprotic conditions were used to stabilize the superoxide ion so that it would not dismutate to H_2O_2. Since an aprotic and not an aqueous environment was used, the significance of these reactions for biological systems remains

to be determined. In addition, it has also been observed that oxygen reacts with metal surfaces of zinc at extremely low temperatures (77K) (88). The molecular steps determined for the dissociative chemisorption of oxygen at the metal surface is as follows: $O_2(g)$ ——> $O_2(a)$ ——> $O^-(a)$ ——> $O^{2-}(a)$ (88). Again, since these reactions were done at extremely low temperatures, the biological relevance is unknown. Several other metals (iron, copper, manganese, and cobalt) are known to react with oxygen (89). These metals are more reactive with oxygen than zinc since they have unfilled d-shell orbitals. Previously, zinc was thought to be unreactive with oxygen because zinc has a filled d-shell orbital (d^{10}). However, a zinc metalloporphyrin is reactive with superoxide and forms a superoxo complex (89). Superoxide has a relatively high affinity for Zn-meso-tetraphenylporphyrin [Zn(TPP)] at 25°C, since it competes with Me_2SO for binding even when Me_2SO is the solvent (89). Zn(TPP) binds only one axial ligand electrostatically since zinc is a d^{10} metal. The reactions of O_2^- with Zn(TPP) are as follows: Zn(TPP) ——> $[Zn(TPP)O_2]^-$ ——?——> $[Zn(TPP)OH]^-$ (117). Perhaps, some form of zinc or complex containing zinc will, in the future, prove to have biological reactivity with oxygen species that participate in the oxygen burst.

In summary, zinc may participate at many steps in production of toxic oxygen metabolites by macrophages. It may be important in the stimulation of NADPH oxidase via phospholipase A_2 or phopholipase C - diacylglycerol lipase catalyzed release of 20:4 from phospholipids. Zinc is an important cofactor for phospholipase C. Zinc may also stabilize 20:4 against oxidation in a Fe-20:4-O_2 complex and thus

allow 20:4 to stimulate NADPH oxidase. Perhaps, zinc or some yet unknown complex containing zinc will prove to have biological reactivity with oxygen or its metabolites. Zinc deficient macrophages which have a reduced ability to destroy T. cruzi would hopefully provide an opportunity to pursue identification of the important zinc dependent processes in the "oxygen burst".

B.) Possible Alterations in the "Oxygen Burst" of Macrophages from Zinc Deficient Mice. The toxic oxygen metabolites of the "oxygen burst" are important in the destruction of certain pathogens including T. cruzi. If zinc is important for normal production of these metabolites, one would expect abnormal functioning of the oxygen burst with zinc deficiencies. This is the case. As discussed in section I C above, zinc deficient mice are unable to combat T. cruzi infections (39). In vitro the zinc deficient macrophage is unable to destroy the T. cruzi (36, 37). Also, addition of zinc but not other metals restored the ability of macrophages from zinc deficient mice to destroy T. cruzi (36, 37).

H_2O_2 produced by macrophages is thought to be important in the destruction of T. cruzi (40, 90). It has been shown that T. cruzi are killed by H_2O_2 (40) and the ability of macrophages to kill T. cruzi correlates with the level of H_2O_2 produced by macrophages stimulated with phorbol 12-myristate 13-acetate (PMA) (91). Catalase, a scavenger of H_2O_2, abrogates the ability of the macrophages to kill T. cruzi (90, 91). In addition, scavengers of other oxygen metabolites such as the superoxide scavenger, superoxide dismutase,

the hydroxyl radical scavengers, mannitol and sodium benzoate, and the possible scavenger of singlet oxygen ($^1O_2$), histidine, do not affect the ability of macrophages to kill T. cruzi (90, 91). In fact, T. cruzi itself contains superoxide dismutase (92) but not catalase (92-94). Thus it is unable to scavenge the H_2O_2. Further evidence for the role of H_2O_2 in killing T. cruzi includes cytochemical indentification of H_2O_2 in polymorphonuclear leukocyte vacuoles containing T. cruzi (40).

Despite this evidence for the importance of H_2O_2 in the destruction of T. cruzi, the amount of H_2O_2 produced by T. cruzi infected macrophages has never been directly measured. It is also important to use T. cruzi as the stimulant when investigating the ability of zinc deficient resident macrophages to produce H_2O_2 since other agents such as PMA probably use different receptors and/or mechanisms of stimulation. Further with regards to association and phagocytosis, it is also important to use T. cruzi rather than other stimulants since resident macrophages from zinc deficient mice are able to phagocytose normal numbers of latex beads (33) but not T. cruzi (36, 37).

Also in the past, it has not been possible with the available assays to detect H_2O_2 produced by resident macrophages (95-99). Usually, macrophages that have already been activated in vivo are used for measuring H_2O_2 production in order to obtain detectable levels of H_2O_2. A modification of the Pick and Mizel assay that can be used to detect H_2O_2 production by unactivated resident macrophages will be described in Chapter 3. As a result of the modification

made, the amount of H_2O_2 produced by resident macrophages stimulated with T. cruzi can now be measured (Chapter 3, 4, 5).

A reduction in the "oxygen burst" would in part explain the reduced ability of zinc deficient animals to combat infections. Since H_2O_2 production is important for destruction of T. cruzi, H_2O_2 production by zinc deficient and zinc adequate macrophages was examined in Chapter 5. The mechanism of stimulation of the "oxygen burst" by T. cruzi is unknown. However, if it is through release of 20:4 and 20:4 stimulation of NADPH oxidase (Figure 2), zinc could be crucial at several steps in the mechanism. As already discussed in the previous section II A, 20:4 levels in phospholipids, the release of 20:4, or the ability of 20:4 to stimulate NADPH oxidase may be altered in zinc deficient macrophages. If there is a reduction in level of 20:4 in deficient macrophages, one would expect reduced stimulation of NADPH oxidase and ultimately a reduced level of H_2O_2 production by the deficient macrophages. 20:4 levels in other tissues from zinc deficient animals are altered. The level of 20:4 in skin is influenced by the level of zinc (79). Also, in the liver of zinc deficient rats, there is a reduced level of 20:4 (100-106). This reduction in 20:4 is accompanied by an increase in cis-linoleic acid, a 20:4 precursor, as a result of a reduction in delta-6-desaturase activity (Figure 3) (100, 107). Delta-6-desaturase is the enzyme which catalyzes the conversion of cis-linoleic acid to gamma linolenic acid. Gamma-linolenic acid is the immediate precursor to dihomogamma linolenate which is then converted to 20:4 by delta-5-desaturase. Clejan et. al. (104) and Field et. al (105) reported

that delta-5-desaturase activity of the liver is also reduced (50-70% of control) in zinc deficient rats. Horrobin and Cunnane (107) have also suggested that zinc may play some role in the mobilization of 20:4 and in delta-5-desaturase activity (Figure 3). Perhaps, zinc deficient macrophages also contain less 20:4 and more of the fatty acid precursors for 20:4. If so, the deficient macrophages would contain fatty acids of shorter chain length and higher degrees of saturation than zinc adequate macrophages. The fatty acid chain length and degree of saturation have been shown to determine the level of superoxide production by NADPH oxidase (48, 108); longer chain fatty acids with higher degrees of unsaturation stimulate production of larger amounts of superoxide. Therefore, upon stimulation of zinc deficient macrophages with T. cruzi, there would be release of shorter-chain more-saturated fatty acids and consequently less stimulation of NADPH oxidase as compared to zinc adequate macrophages. Thus, there would most likely be a reduction in H_2O_2 production and in turn reduced killing of T. cruzi. The results of fatty acid analysis of phospholipids from zinc deficient and zinc adequate macrophages is presented in Chapter 5.

Besides a possible reduction in synthesis and incorporation of 20:4 into phospholipids of zinc deficient macrophages, there may also be a reduction in release of 20:4 from the phospholipids. As discussed earlier in section II A , zinc may be important in the release of 20:4 from phospholipids since phospholipase C is a zinc dependent enzyme (Figure 2) (69, 70). Depletion of zinc from the enzyme would reduce phospholipase C activity which, in turn, would

reduce the amount of 20:4 released for stimulation of NADPH oxidase resulting ultimately in less H_2O_2 production. Also, a reduction in the stimulation of the release of 20:4 may in part be due to the reduced number of T. cruzi associated with the zinc deficient macrophages. So, zinc may play a role in the events of binding and/or stimulation of macrophage H_2O_2 production.

If there is a reduction in 20:4 in or released from plasma membranes of zinc deficient macrophages, there would probably also be a reduction in production of 20:4 metabolites of the cyclo-oxygenase and/or lipoxygenase pathways. It has been reported that lipoxy--oxygenase but not cyclo-oygenase products stimulate NADPH oxidase (54). However, lipoxygenase products are not absolutely required for stimulation of NADPH oxidase since lipoxygenase inhibitors do not affect the "oxygen burst" (54). Most likely, with inhibition of lipoxygenase, the lipoxygenase substrate 20:4 stimulates NADPH oxidase. To further demonstrate the importance of lipoxygenase products in the "oxygen burst" and the killing of T. cruzi, Wirth et. al. (109, 110) showed that addition of leukotriene B_4 or leukotriene C_4, lipoxygenase products, increased killing of T. cruzi and that this effect of the leukotrienes is abrogated by the H_2O_2 scavenger catalase (109. 110). Since leukotrienes can stimulate the "oxygen burst" (60), perhaps, the increase in killing of T. cruzi was the result of increased stimulation of H_2O_2 production by leukotrienes. If leukotriene production is reduced in the zinc deficient macrophages, this could also explain the reduction in the association with and H_2O_2 mediated destruction of T. cruzi by zinc deficient macrophages.

Once 20:4 is liberated from the macophage phospholipid, zinc may compete with iron for complex formation with 20:4 and oxygen (78). Since the complex containing iron but not zinc oxidizes 20:4, zinc may stabilize 20:4 against oxidation. In the case of the zinc deficient macrophage, perhaps, a larger portion of the 20:4 is oxidized by the iron complex and, as a consequence, less 20:4 is available for stimulation of NADPH oxidase. Thus, there would be less production of O_2^- and consequently H_2O_2 by deficient macrophages. The analysis of the release of 20:4 and its metabolites upon stimulation of zinc deficient macrophages with T. cruzi is presented in Chapter 5. In summary, if levels of 20:4 released by zinc deficient macrophages is reduced, it may be due to reduced synthesis of 20:4 for incorporation into phospholipids, reduced stimulation for release of 20:4 from phospholipids, or increased oxidation of the released 20:4.

Still, there exists other possibilities for a reduction in the "oxygen burst" of zinc deficient macrophages. Unfortunately these reactions are not well defined. For example, a reduction in H_2O_2 production could be due to reduced availability of zinc for catalysis of some as yet unknown chemical reactions with oxygen. Or, zinc may be unavailable for the formation of some zinc nucleotide complexes other than Zn_2-NADPH that perhaps participate in the "oxygen burst". Also, it remains to be determined whether or not any of the known mechanisms for stimulation of the "oxygen burst" by macrophages hold true when T. cruzi is the stimulant. Even though not much is known about T. cruzi-macrophage interactions or the importance of zinc in the "oxygen burst", the literature discussed in this chapter

implicates many speculative roles for zinc in the "oxygen burst". Much work must be done to elucidate the role(s) for zinc in the production of toxic oxygen metabolites by macrophages as well as the defect of zinc deficient macrophages in associating with and destroying the pathogen, T. cruzi.

C.) Possible Alterations in Membranes of Macrophages from Zinc Deficient Mice. Zinc may also be important in the association of T. cruzi with the macrophage since, as discussed in section I C, association with zinc deficient macrophages is reduced (36, 37). Additon of zinc repairs this defect (36, 37). Zinc has been shown to play a role in stabilization of membranes against perturbing agents or oxidations. Zinc is also necessary for synthesis of some important components of the membrane such as 20:4. Whether these or other interactions between zinc and the plasma membrane are altered in the zinc deficient macrophage remains to be determined.

Since zinc has been shown to stabilize membranes, perhaps, this trace element is also important for association of the parasite with the macrophage membrane. The evidence for stabilization of membranes by zinc is far from complete. So far, it has been shown that plasma membranes contain substantial amounts of zinc (79), that supra-physiological levels of zinc stabilizes membranes against perturbing agents (79), and that sub-physiological levels of zinc leads to loss of plasma membrane zinc and destabilization of the membrane (79). The level of zinc (64-222 μg/g protein) in the plasma membrane (111) may have considerable physiological relevence since the plasma membrane

has rather diverse functions including transport of nutrients, maintenance of osmolarity, and stimuli transduction through receptors. Although the role(s) for zinc in the membrane has not been delineated, zinc has been found to be associated with several membranes (plant membranes, plasma and lysosomal membranes of lung alveolar macrophages, lysosomal membranes of liver cells, neurotubules, microtubules, and plasma membranes of erythrocytes (79). A substantial level of zinc is in the membranes of erythrocytes (115 $\pm$ 9.7 μg/g phospholipid) (111). A similar amount of another metal, copper, is associated with the erythrocyte membrane (128 μg/g phospholipid) (111). The zinc in erythrocyte plasma membranes is mainly associated with the lipid phase (69% of the zinc), in particular the phospholipids, with some zinc bound to membrane proteins (111). As stated earlier, zinc may stabilize 20:4 from oxidation in the iron-20:4-oxygen complex (78). Zinc has been shown to prevent lipid peroxidation of 20:4 in liver microsomes *in vitro* (79). Chvapil et. al. (111) speculate that this is due to an interaction of zinc with polyunsaturated fatty acids of phospholipids or with proteins of membranes. Also, high dietary zinc inhibits *in vitro* induction of lipid peroxidation of liver microsomes (79). Thus, addition of zinc ions seem to inhibit lipid peroxidation of biological membranes (79). In fact, high levels of zinc are used to stabilize various membranes during membrane purification procedures (79).

Membrane integrity is altered by a deficiency in zinc. Histology of membranes from several zinc deficient animals, including rat pancreas and intestinal cells, tumor cells, and *Euglena gracilis*,

showed alterations in membrane morphology (73). In the zinc deficient state, the chick embryo is unable to maintain normal ionic balance or cell volume (79). Erythrocytes from zinc deficient rats are more prone to hemolysis and this correlates with the level of reduced serum zinc (79). In addition, when zinc was added to normal erythrocytes, they acquired an increased stability against osmotic shock (79). Zinc deficiencies also have adverse effects on leukocyte permeability to sodium (73). Thus, it is possible that pretreatment of zinc deficient macrophages with zinc may stabilize the macrophage membranes making possible normal levels of association of the parasites. In turn, there would be normal stimulation of the "oxygen burst" since the level of T. cruzi-macrophage association correlates with T. cruzi stimulated H_2O_2 production by resident macrophages (Chapter 4).

Another explanation for the reduced association of T. cruzi with zinc deficient macrophages may be alterations in the fatty acid composition of the macrophage membrane. If zinc deficient macrophages have a reduction in the more unsaturated fatty acid, 20:4, and an increase in more saturated precursors, this may lead to a reduction in phagocytosis and possibly the decrease in association of T. cruzi seen with zinc deficient macrophages. It has been reported that a reduction in unsaturated fatty acids and a concomitant increase in more saturated fatty acids leads to reduced fluidity of the membrane bilayer, reduced phagocytosis, and increased activation energy for phagocytosis in macrophages (112, 113). As discussed in section II B, deficiencies in zinc reduce the level of 20:4 in the

rat liver (100-106). This reduction was accompanied by an increase in cis-linoleic acid, a precursor to 20:4, due to reduced activity of delta-5- and delta-6-desaturase (100, 104, 105, 106) (Figure 3). These alterations may also occur in the deficient macrophages. If the fatty acid composition is altered in zinc deficient macrophages, one would expect that a 30 minute incubation with zinc would restore the plasma membrane fatty acid composition to normal since T. cruzi-macrophage association is restored. The possible restoration of fatty acid composition in the presence of zinc may occur during recycling of the plasma membrane of zinc deficient macrophages. The resident macrophage has an extremely high rate of plasma membrane pinocytosis; the entire membrane is endocytosed approximately every 33 minutes (114). The majority of this endocytosed membrane is recycled to the cell surface (115, 116). Unfortunately, the mechanisms for membrane recycling are not well understood (115). However, it is thought that the golgi apparatus (115-118) and the endoplasmic reticulum (118) may be involved in this recycling process. Exposure of the internalized membrane to these organelles during recyclying may be important for repair of membrane components especially after exposure to the degredative processes occuring in the phagolysosome. Since fatty acid elongation and desatuation normally occur in the endoplasmic reticulum and these processes may be decreased in deficient macrophages, perhaps, the fatty acid composition of the membranes of zinc deficient macrophages can be significantly restored by the membrane recycling processes during the consecutive incubations of a half hour with zinc and one hour with T. cruzi. To repair

deficient macrophages, modifications of existing fatty acids is more probable than synthesis of new fatty acids since synthesis requires several hours (118) yet repair occurs within 1 1/2 hours (36, 37). One process of phospholipid synthesis/repair has been shown to occur rather rapidly during phagocytosis; phosphatidylinositol turnover as measured by P^{32} incorporation can be detected in 10 minutes with a maximal rate between 1 and 6 hours (119). In summary, the reduced association of T. cruzi with zinc deficient macrophages may be due to an altered fatty acid composition of the plasma membrane and these alterations may be repaired by addition of zinc. The fatty acid composition of the phospholipids from zinc deficient and zinc sufficient macrophages was determined and will be discussed in Chapter 5.

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Chapter 2

FUNCTIONAL CAPACITY OF THE RESIDUAL LYMPHOCYTES IN ZINC DEFICIENT MICE

ABSTRACT

Zinc deficiency, a condition not infrequently encountered in humans, drastically reduces cell and antibody mediated responses. Since there are numerous zinc dependent metalloenzymes necessary for lymphocyte and macrophage function, it was of interest to determine the capacity of the residual lymphocytes from zinc deficient mice to proliferate and produce lymphokines in response to stimulation. In these studies, minimal levels of zinc were introduced *in vitro* by using autologous serum from zinc deficient mice or a serum free system. Splenocytes from moderately or severely zinc deficient adult A/J mice gave normal proliferative responses and generated adequate interleukin 2 (IL-2) activity when stimulated with Concanavalin A in a serum free system. When stimulated with allogeneic target cells, splenocytes from deficient mice exhibited enhanced proliferation and IL-2 production (180%) compared to the splenocytes from adequately fed mice regardless of whether the amount of zinc in the culture medium was ample or limiting. The increased proliferation was probably not due to a lower threshold for stimulation since the number of IL-2 receptors per cell from the deficient mice was the same as controls. However, more cells were available for stimulation in the deficient group since a greater proportion of cells bore IL-2 receptors. Although B-cells from zinc deficient mice stimulated *in vivo* with sheep red blood cells produced fewer total numbers of plaque forming cells (PFC) per spleen, the proportion of PFC per 10^6 viable splenocytes and the amounts of IgM and

IgG produced per plaque were normal. Based on the tests performed thus far, ample or limiting amounts of zinc in the culture medium do not affect lymphocyte responses and it would appear that the residual splenic lymphocytes of zinc deficient mice are able to carry out many fundamental immune processes.

INTRODUCTION

Zinc deficiency is a prevalent human nutritional problem throughout the world, including the USA (1-4). In both man and animals, zinc deficiency causes rapid and severe depressions in immune function (5). In the mouse, zinc deficiency causes drastic reductions (40-50%) in the absolute numbers of lymphocytes and macrophages in the blood, thymus, and spleen (5, 6). Yet, the relative percentage of T- and B-cells and mononuclear cells remained unchanged (5, 6). Antibody mediated responses to both thymus independent and thymus dependent antigens in mice show marked depression (50-70%) depending on the degree of the deficiency (5, 7, 8, 9, 10, 11). Other T-cell dependent responses such as delayed-type hypersensitivity and cytolysis of tumor cells are also substantially reduced by the deficiency (12, 13).

Because of the greatly reduced capacity of zinc deficient mice to mount immune responses the question arose as to whether the depression in immune function was due only to decreased numbers of lymphocytes and/or to a decrease in functional capacity of the residual lymphocytes. There are over 100 enzymes that are dependent upon zinc for function including many enzymes associated with RNA and DNA synthesis (2). Suboptimal dietary zinc might, therefore, affect the activity of these enzymes and other zinc dependent processes thereby altering proliferation and production of lymphokines by lymphocytes. For this reason, the ability of T and B-cells from zinc deficient mice to respond to various stimulants was assessed along with their

capacity to proliferate and produce interleukins and antibodies. Care was also taken to regulate the level of exogenous zinc in the culture medium. To this end, lymphocytes were cultured in medium supplemented with sera from zinc deficient mice or in a serum free system containing concentrations of zinc that were below physiological levels to reduce the possibility of *in vitro* rejuvenation of zinc dependent processes. These results were compared to data obtained with cultures supplemented with fetal calf serum which contains significant levels of bioavailable zinc. The results will show that lymphocytes from zinc deficient mice are able to perform many key immune functions even in those culture systems where the availability of zinc was limited.

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MATERIALS AND METHODS

Animals. A/J and C57Bl/6 female mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Female Lewis rats were purchased from Charles River Breeding Laboratories, Portage, Michigan.

Diets. Six week old A/J female mice were placed in stainless steel cages with mesh bottoms to reduce recycling of zinc. They were fed ad libitum a biotin fortified egg white diet containing either deficient (0.8μg Zn/g) or adequate (27μg Zn/g) levels of zinc. The composition of the diet is described in detail in Appendix. Since inanition accompanies zinc deficiency, a third group, the restricted mice, were fed zinc adequate diet equivalent to the average amount of food consumed the previous day by zinc deficient mice. All mice had free access to deionized distilled water (<0.2μg Zn/g). Feed jars and water bottles were washed with 4N HCl and rinsed with deionized water to remove zinc. The mice were weighed at least once a week. At the end of the dietary period, those mice that received zinc deficient diet and weighed 65-68% of the average body weight of the control mice were designated as severely zinc deficient mice. Moderately deficient mice were defined as weighing 70-74% of the average control mouse body weight. Previous studies indicated the latter group is only modestly effected by inanition (8). The total degree of parakeratosis per mouse was determined by summing the degree of parakeratosis assigned on a scale of 0 to 4 to the eye, tail, ears, anus, and coat. Thymuses were also weighed for each dietary group.

Collection of autologous serum. Serum was collected by severing the subclavian artery of severely zinc deficient or control mice. The blood was incubated for 15 minutes at 37°C followed by several hours at 4°C. Mouse serum (0.5% v/v) supplemented culture medium was filter sterilized using a 0.2 micron Nalgene filter.

Zinc analysis. The diets and sera were analyzed for zinc content by atomic absorption spectrophotometry (Varian Techron AA-175, Springvale, California) as described in earlier publications (7, 14).

Cell Culture. The culture medium consisted of RPMI-1640 (M.A. Bioproducts, Walkersville, MD) buffered with 0.01M Hepes pH 7.4 (Gibco Laboratories, Grand Island, N.Y.), 0.06%w/v $NaHCO_3$ (Gibco), and supplemented with 0.1mM nonessential amino acids (M.A. Bioproducts), 2mM glutamine, 1mM Na pyruvate (M.A. Bioproducts), 100 units penicillin, 100µg/ml streptomycin (M.A. Bioproducts), 50 µg/ml gentamycin (M.A. Bioproducts), 5 x 10^{-5}M 2-mercaptoethanol, and 0.1X BME vitamin mix (M.A. Bioproducts) (15). The medium was supplemented with 0.5%v/v serum collected from deficient or control mice and 0.8%w/v bovine serum albumin (tissue culture grade BSA, Sigma, St. Louis, MO), or with 5%v/v fetal calf serum (FCS, M.A. Bioproducts). Spleens were removed asceptically, minced, and pressed through a sterile stainless steel mesh (100 gauge). The single cell suspension was washed and cell viability was determined using the trypan blue dye exclusion method. Cultures were incubated at 37°C under a humidified atmosphere of 10% CO_2, 7%O_2, and 83% N_2 or 7% CO_2 and ambient air.

Mitogenic stimulation. Splenocytes (2.5 x 10^5) were cultured in 96 well flat bottom microtiter plates (Falcon Plastics, Oxnard, CA) in serum free medium containing Concanavalin A, (Con A, Sigma), at concentrations ranging from 0.5 to 5 μg/ml. After 24 hours, 1μCi of methyl-[^{3}H]-thymidine (2 Ci/mM, Amersham) was added to each well. Eighteen hours later, the cultures were harvested using a multiple sample harvester (Otto Hiller Co., Madison, WI), and the DNA was precipitated with cold trichloroacetic acid onto glass fiber filters. The amount of radioactivity incorporated into the DNA was measured by a Delta 300 scintillation counter (Tracor Analytic). Unstimulated A/J splenocytes incorporated less than 10,000 cpm of methyl-[^{3}H]-thymidine. At the time of harvest, cell viability, as determined using the trypan blue exclusion method, was 70% ± 6%. Also, 24 hour supernatants from Con A stimulated A/J splenocytes were collected for analysis of interleukin-2 (IL-2) activity.

Mixed lymphocyte culture (MLC). The culture medium described above was supplemented with either 0.5% autologous serum from zinc deficient mice (50 μg Zn/dl) or control mice (100 μg Zn/dl) and 0.8% BSA (tissue culture grade) or with 5% FCS. In order to render the target cells unresponsive, splenocytes from C57Bl/6 (H-2^b) mice were incubated with mitomycin C (25 μg/ml) for 30 minutes. 2.5 x 10^5 A/J splenocytes (H-2^a) were incubated with 1 x 10^5, 2.5 x 10^5, or 7.5 x 10^5 mitomycin C treated C57Bl/6 splenocytes (H-2^b). Methyl-[^{3}H]-thymidine was added for the final 18 hours of a 3, 4, 5, or 6 day incubation period. The level of radioactivity incorporated into the DNA was determined as described for the Con A assay. Mitomycin C

treated C57Bl/6 splenocytes incorporated less than 1000 cpm of methyl-[^{3}H]-thymidine. At the time of harvest, A/J splenocyte viability as determined by trypan blue exclusion was 91 $\pm$ 4%. In addition, supernatants were collected and pooled from identically stimulated cultures (triplicates) at 48 hours for analysis of IL-2 activity. The ability of mitomycin C treated cells to absorb, produce, or proliferate in response to IL-2 was also analyzed. Absorbtion of IL-2 by mitomycin C treated cells was determined by adding IL-2 to mitomycin C treated cells and measuring IL-2 activity in 24 hour supernatants. Production of IL-2 was determined by measuring IL-2 activity in 24 hour supernatants from mitomycin C treated A/J and mitomycin C treated C57Bl/6 cells. Proliferation in response to IL-2 was determined from methyl-[^{3}H]-thymidine incorporation by mitomycin C treated cells supplemented with IL-2.

Assay for IL-2 activity. CTLL-2 cells (1 x 10^4), an IL-2 dependent cytolytic T-cell line (Scripps Clinic, La Jolla, CA), were supplemented with various dilutions of standard or test supernatants from either Con A or allogeneic cell-stimulated splenocytes so that the final culture volume was 100 µl. Supernatants from the zinc adequate splenocytes served as the standard. The cultures were pulsed with 1 µCi methyl-[^{3}H]-thymidine for the final 18 hours of a 42 hour incubation followed by determination of the amount of methyl-[^{3}H]-thymidine incorporated into the DNA. One unit/ml of IL-2 activity was assigned to that dilution of the supernatant from zinc adequate splenocytes which induced 50% of the maximum

methyl-[^{3}H]-thymidine incorporation. IL-2 activity was quantified by probit analysis (16).

Maintenance of cytolytic T-cell line. CTLL-2 cells (5 x 10^3/ml) were cultured in IL-2 supplemented culture medium (50%/50% v/v) and subcultured every 2-3 days when saturation density (approximately 5 x 10^5 to 10^6 CTLL-2 cells/ml) was reached. IL-2 supernatants to maintain this cell line were obtained from Con A (2 μg/ml) stimulated female Lewis rat splenocytes (1 x 10^6 cells/ml) after 48 hours of culture.

IL-2 receptors. IL-2 receptors on splenocytes were labeled by indirect immunofluorescense using 7D4, a rat IgM monoclonal antibody which is specific for the IL-2 receptor (17, 18). Cells from each group were collected on day 3, 4, 5, or 6 of the MLC, pooled and placed over a lympholyte M gradient (Accurate Chemical and Scientific Corp.). The lymphocytes in the interface of the gradient were collected and washed three times with 0.1M phosphate buffered saline (PBS, pH 7.4) - 0.15% BSA - 0.15% NaN_3. Viable lymphocytes (1 x 10^6) were placed on ice and resuspended in 200 μl of 7D4 antibody with 0.15% NaN_3 for 30 minutes at 4°C. The cells were washed three times and resuspended in 100 μl of PBS-BSA-NaN_3 followed by the addition of 70 μl of a 1/2 dilution of fluorescein isothiocyanate (FITC) conjugated affinity purified mouse anti-rat kappa (FITC-Mar 18.5, Becton Dickinson). After a 30 minute incubation at 4°C, the cells were washed three times in the cold with PBS-BSA-NaN_3 and resuspended to 2 ml. The relative number of IL-2 receptors per cell and the percentage of cells with receptors was analyzed using an

Orthocytofluorograph 50-H connected to a 2150 computer (Ortho Diagnostic Systems Inc., Raitan, N.J.) The percentage of fluorescently labelled cells and cellular debris was obtained from a cytogram of forward scatter versus 90 degree angle scatter of the fluorescently labelled material. Less than 15% fluorescence was cellular debris. A background of less than 3% fluorescence was observed when FITC-MAR18.5 antibody was added alone. The data is shown as the percentage of cells, excluding cellular debris, that were fluorescently labelled.

Antibody production. A/J mice in each dietary group were immunized intraperitoneally with 1 x 10^8 SRBC in sterile PBS. Five days later, spleens were removed for the determination of the number of immunoglobulin secreting cells and the average amount of immunoglobulin secreted per cell. The number of direct (IgM) and indirect (IgG) anti-SRBC plaque forming cells (PFC) from each spleen was determined using a modification of the Jerne plaque assay as described in detail elsewhere (19). A nonimmunized mouse, included as a control, produced less than one PFC per million spenocytes. For the determination of the average amount of immunoglobulin produced per PFC, 10 x 10^6 splenocytes were suspended in 1 ml of culture medium supplemented with 1% gamma globulin free BSA (Calbiochem) and placed in a 12 x 75mm culture tube (Falcon) for two hours at 37°C and 7% CO_2. Supernatants were collected and the amount of IgG and IgM produced was determined by radioimmunoassay as described below.

Radioiodination. Mouse gamma-globulin (Calbiochem), and mouse IgM (MOPC 104E, Bionetics) were radioiodinated in the presence of Iodogen, as previously described (20). Specific activity of the

iodinated immunoglobulin was 1.3 x 10^6 cpm/μg for IgM and 2.1 x 10^5 cpm/μg for IgG.

Radioimmunoassay. The amount of IgM or IgG produced in 2 hours by cultured splenocytes from mice immunized with SRBC was determined by radioimmunoassay. Supernatants to be tested or unlabeled immunoglobulins to be used as standards (mouse IgM, MOPC 104E, Bionetics or mouse IgG, Calbiochem) and 5 x 10^3 μg of radioiodinated IgM or IgG were added to 10 μl of normal rabbit serum, along with either affinity purified rabbit anti-mouse μ or rabbit anti-mouse gamma (Zymed). The antibody complexes were allowed to form for 1 hour at 37°C. Then, the complexes were precipitated with 90 μl of goat anti-rabbit serum, which had been adsorbed with mouse immunoglobulins. The precipitate was washed twice with borate buffered saline (pH 8.0) - 1%w/v BSA, dissolved in 0.1N NaOH, and counted in a gamma counter. There was no cross reactivity as detected by this assay for mouse IgM with rabbit anti-mouse gamma or for mouse IgG with rabbit anti-mouse μ. The amount of immunoglobulin in the test supernatant was determined from a standard curve using known amounts of IgM or IgG. The average amount of IgG or IgM produced per IgG or IgM plaque forming cell (PFC) was calculated as follows:

Amount of immunoglobulin produced by 10^6 cells
divided by PFC/10^6 cells =
average amount of immunoglobulin produced/PFC

For splenocytes from nonimmunized mice, antibody production per PFC was zero as determined by this assay.

Statistics. The mean and standard error of the mean were calculated for each treatment group. Probability values for the comparison of the zinc deficient and restricted groups to the control group were determined by a completely random ANOVA followed by Dunnett's t Test.

RESULTS

The effects of a zinc deficient diet on growth, diet consumption, thymus weight, degree of parakeratosis, serum zinc and splenocyte numbers are shown in Table 1. After a 30 day dietary period, the zinc deficient mice had consumed 12% less diet than the control mice. The average severely zinc deficient mouse selected for study weighed 66% of that of control mice, while the moderately zinc deficient mice weighed 72% of that of control mice. Past data indicates that inanition will have a greater effect on the lower than the higher weight group with regard to T-cell dependent antibody mediated responses and thymus weights (8). The restricted mice, which consumed the same amount of diet as the deficient mice, weighed 82% of that of control mice. The degree of parakeratosis for the severely zinc deficient mice was about twice that of moderates (Table 1); restricted and control mice showed no signs of parakeratosis. The thymus weights of the severe mice were significantly smaller than the moderates which were significantly lower (approximately 50% of controls) than the restricted or control mice (Table 1). There was no difference between the thymus weights of the restricted and control mice. In addition, the average number of cells per spleen from the severely and moderately zinc deficient mice was reduced by 43% and 47%, respectively, compared to zinc adequate mice. However, the number of splenocytes in the restricted group was reduced by only 17% compared to controls. Sera collected from the severely and moderately deficient mice to test zinc status and for use in culture contained 50

TABLE 1

Body Weight, Diet Consumption, Degree of Parakeratosis, Thymus Weights, and Serum Zinc Levels of Mice After 30 Days on Zinc Deficient or Zinc Adquate Diet

Dietary Group	Severely Zinc Deficient	Moderately Zinc Deficient	Restricted	Zinc Adequate
Initial Body Wt. (g)	16.9 ± 0.07[a]	16.9 ± 0.07	17.0 ± 0.25	17.1 ± 0.15
Final Body Wt. (g)	14.3 ± 0.52[b]	15.8 ± 0.15[b]	17.7 ± 0.05[b]	21.8 ± 0.20
Food Consumption (g)	73.4 ± 4.45[b,c]		73.4 ± 4.45[b]	92.7 ± 4.82
Degree of Parakeratosis[d]	8.3 ± 1.2[b]	3.1 ± 0.4[b]	0.0 ± 0.0	0.0 ± 0.0
Thymus Wt. (g)	6.5 ± 0.8[b]	17.6 ± 1.3[b]	30.4 ± 1.3	33.7 ± 1.0
Cells per Spleen ($x10^{-7}$)	1.9 ± 0.06[b]	1.8 ± 0.10[b]	2.7 ± 0.20	3.3 ± 0.25
Serum Zinc Levels (µg/dl)	52.4 ± 1.6[b]	48.5 ± 2.2[b]	101.9 ± 2.7[b]	98.5 ± 2.1

[a] Mean ± SEM of 5 or 8 mice

[b] $p < 0.01$ as compared to zinc adequate mice

[c] Average diet consumption by zinc deficient mice prior to separation into severely and moderately deficient groups

[d] Sum of degree of parakeratosis assigned to eye, tail, ears, coat, and anus on a scale of 1 to 4

μg Zn/dl compared to 100 μg Zn/dl for control mice (Table 1). This was similar to zinc levels reported in previous studies (15).

In the first study, the capacity of residual splenic T-cells of deficient mice to proliferate and produce IL-2 in response to stimulation with Con A was assessed. In this case, the splenocytes were cultured in serum free medium since FCS contains high levels of zinc (350 μg Zn/dl) which, in turn, may initiate in vitro repair by cells from zinc deficient mice. Serum supplementation was not necessary for good cell viability (70 ± 6%) or proliferation in response to Con A. In these experiments the level of zinc in the medium was 8 μg Zn/dl. Optimal splenocyte proliferation by the zinc adequate group was obtained on day 1 (24 hour incubation with Con A plus an 18 hour pulse with ^{3}H Thymidine) (Figure 1). When pulsed on day 1, optimal proliferation for all dietary groups was obtained using 2 μg/ml of Con A (Figure 2). As can be seen from Figure 2, there was no significant difference in the proliferation of splenocytes among the four dietary groups regardless of the concentration of Con A. Optimal IL-2 production was observed at 24 hours after stimulation with 2 μg/ml of Con A (data not shown). From Figure 3, it is apparent that production of IL-2 by the moderately zinc deficient and restricted dietary groups was similar to that by the zinc adequate group. The production of IL-2 by cells from the severely zinc deficient mice though slightly lower than the control group was not significantly different.

Figure 1. Dose curve and kinetics in a serum free system of the proliferative response to Con A by splenocytes prepared from mice consuming normal laboratory chow. On day 1, 2, 3, or 4, ^{3}H-thymidine was incubated with the splenocytes for an additional 18 hours. Incorporation of ^{3}H-thymidine into the DNA was determined. Each point represents the mean $\pm$ SEM of 3 mice.

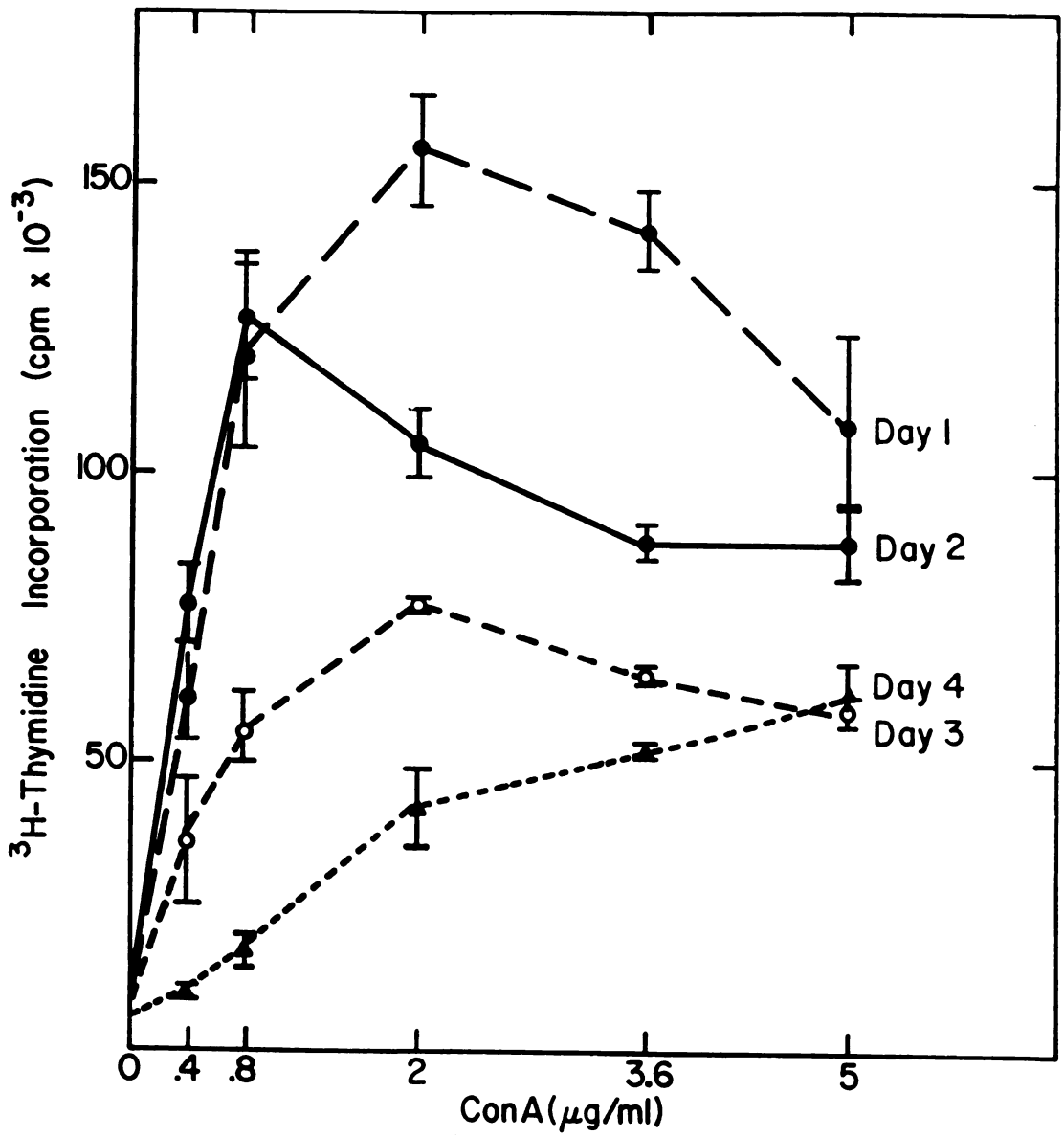

^{3}H-Thymidine Incorporation (cpm x 10^{-3})
150
100
50
0
.4
.8
2
3.6
5
ConA(μg/ml)
Day 1
Day 2
Day 4
Day 3

Figure 2. Dose curve for the proliferative response in a serum free system to Con A by splenocytes prepared from severly zinc deficient, moderately zinc deficient, restricted, and control mice. On day 1, ^{3}H-thymidine was incubated with the splenocytes for an additional 18 hours. Then, incorporation of ^{3}H-thymidine into the DNA was determined. Each point respresents the mean $\pm$ SEM of five or six mice.

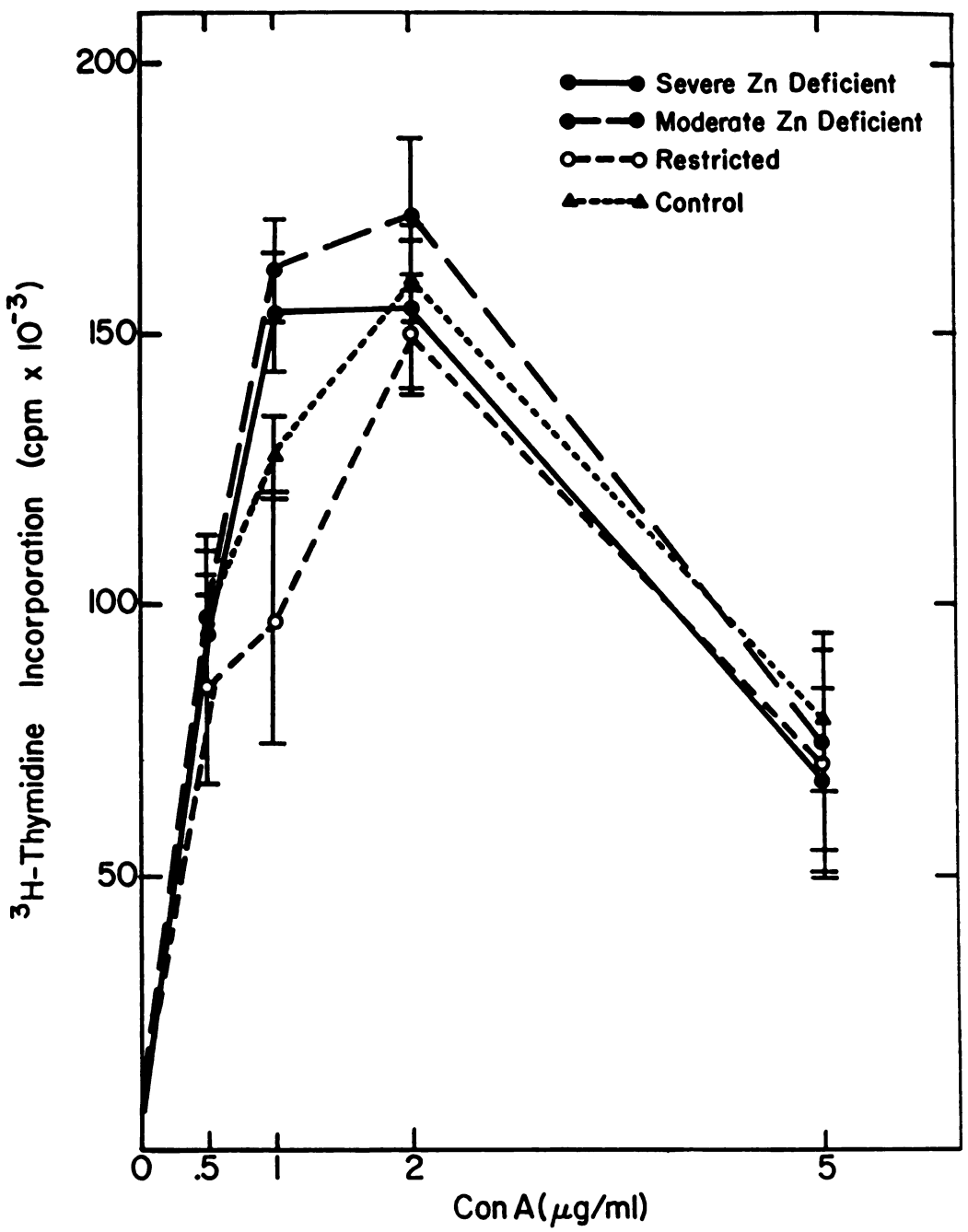
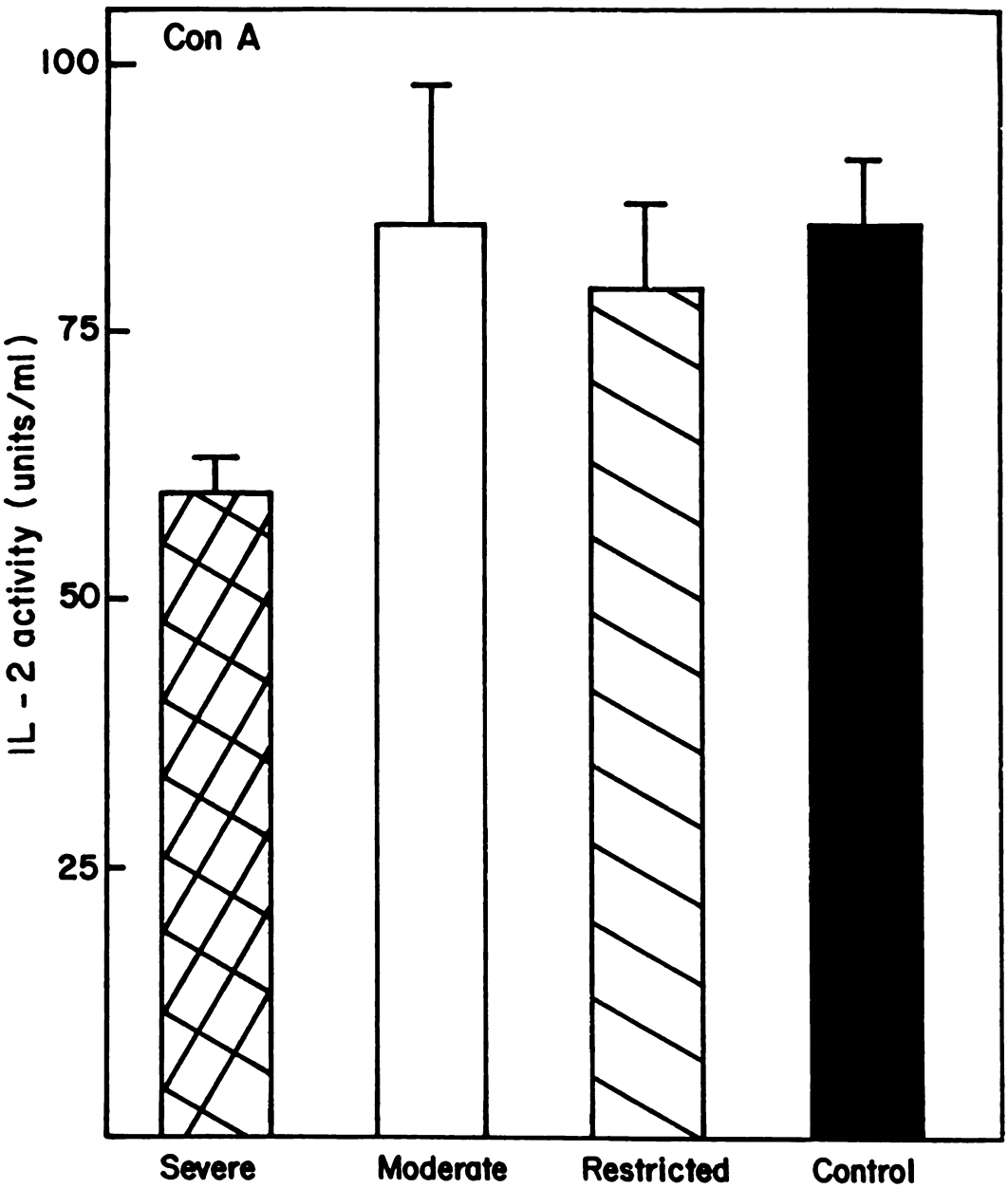

Severe Zn Deficient
Moderate Zn Deficient
Restricted
Control
200
150
100
50
0
.5
1
2
5
^{3}H-Thymidine Incorporation (cpm x 10^{-3})
Con A(μg/ml)

Figure 3. IL-2 activity in serum free culture medium harvested at 24 hours from triplicate cultures with 2 μg/ml Con A-stimulated splenocytes prepared from severely zinc deficient, moderately zinc deficient, restricted, or control mice. IL-2 activity was measured by the assay using the IL-2 dependent CTLL-2 cell line. Each bar represents the mean ± SEM of five or six mice.


Con A
100
75
50
25
IL - 2 activity (units/ml)
Severe
Moderate
Restricted
Control

The ability of residual T_H-cells to respond to allogeneic cells during a MLC was also studied by measuring splenocyte proliferation and IL-2 production. The MLC was supplemented with serum in order to obtain good cell viability and proliferation. In 5% FCS supplemented medium, optimal splenocyte proliferation was achieved for all dietary groups on day 4 (Figure 4) using 7.5×10^5 mitomycin C treated C57Bl/6 stimulator cells. In a subsequent study using 5% FCS, optimal splenocyte proliferation on day 5 was obtained using 2.5×10^5 mitomycin C treated cells (Figure 5). Surprisingly, the zinc deficient groups had significantly higher proliferative responses than the control group; the proliferation by splenocytes from severely and moderately zinc deficient A/J mice were 185% and 142%, respectively, of controls (Figure 5). Since FCS contains high levels of zinc (351 μg Zn/dl), there was concern that the higher response by the deficient groups may have been due in part to the availability of zinc resulting in <u>in vitro</u> repair by the deficient groups. To test this, we minimized the addition of zinc by using 0.8% BSA (0.4 μg Zn/dl) and 0.5% autologous serum from zinc deficient (40 μg Zn/dl) to supplement the culture medium. It should be noted that the RPMI-1640 and the various medium supplements contain only 8 μg Zn/dl. Figures 6 and 7 show the kinetics, and figures 8 and 9 show the dose response in zinc deficient or zinc adequate autologous serum plus BSA. Using BSA and autologous deficient sera (40 μg Zn/dl) or autologous adequate sera (107 μg Zn/dl), the optimal proliferation was to day 5 (Figures 6, 7) using 1×10^5 mitomycin C treated C57Bl/6 target cells (Figures

Figure 4. Kinetics of the MLC proliferative response in 5% FCS supplemented medium. A/J responder splenocytes (2.5×10^5) from severely zinc deficient, moderately zinc deficient, restricted, or control mice were cultured with 7.5×10^5 mitomycin C-treated C57Bl/6 splenocytes and pulsed for 18 hours with ^{3}H-thymidine on day 3, 4, or 5. Each bar represents the mean $\pm$ SEM for five or six mice. Asterisk indicates significance of $p < 0.05$ or better as compared to control responses.

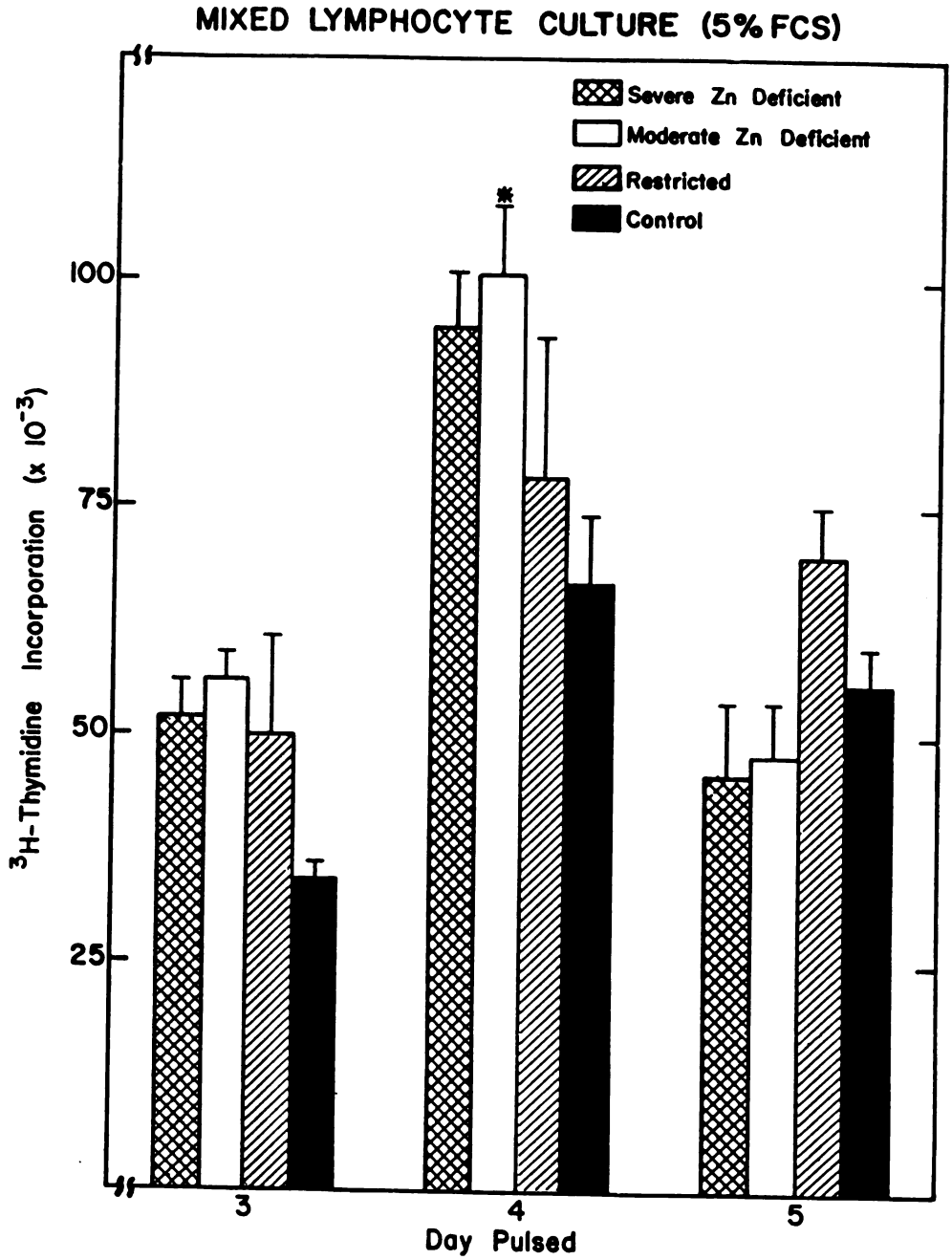

MIXED LYMPHOCYTE CULTURE (5% FCS)
Severe Zn Deficient
Moderate Zn Deficient
Restricted
Control
*
100
75
50
25
^{3}H-Thymidine Incorporation (x 10^{-3})
3
4
5
Day Pulsed

Figure 5. Dose curve for the MLC proliferative response in 5% FCS supplemented medium. A/J responder splenocytes (2.5×10^5) from severely zinc deficient, moderately zinc zinc deficient, restricted, or control mice were cultured with mitomycin C-treated C57Bl/6 splenocytes and pulsed for 18 hours with ^{3}H-thymidine on day 5. Each point represents the mean $\pm$ SEM for five or six mice. Asterisk indicates significance of $p < 0.05$ or better as compared to control responses.

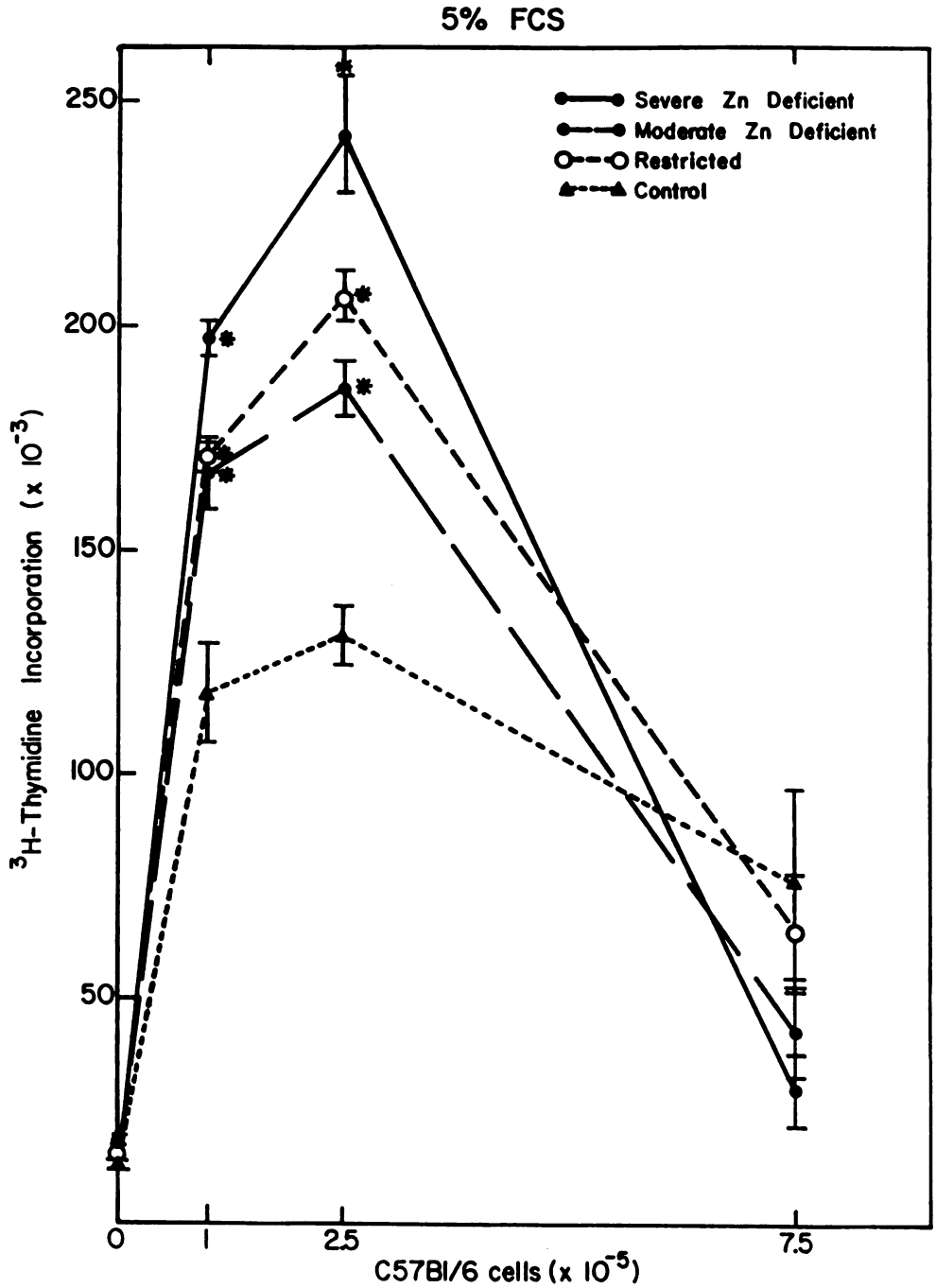

5% FCS
Severe Zn Deficient
Moderate Zn Deficient
Restricted
Control
250
200
150
100
50
0
1
2.5
7.5
^{3}H-Thymidine Incorporation (x 10^{-3})
C57Bl/6 cells (x 10^{-5})

Figure 6. Kinetics of the MLC proliferative response in 0.5% zinc deficient autologous serum supplemented medium. A/J responder splenocytes (2.5×10^5) were cultured with 1×10^5 mitomycin C-treated C57Bl/6 splenocytes and pulsed for 18 hours with ^{3}H-thymidine on day 4, 5, or 6. Each bar represents the mean $\pm$ SEM for five or six mice. Asterisk indicates significance of $p < 0.05$ or better as compared to control responses.

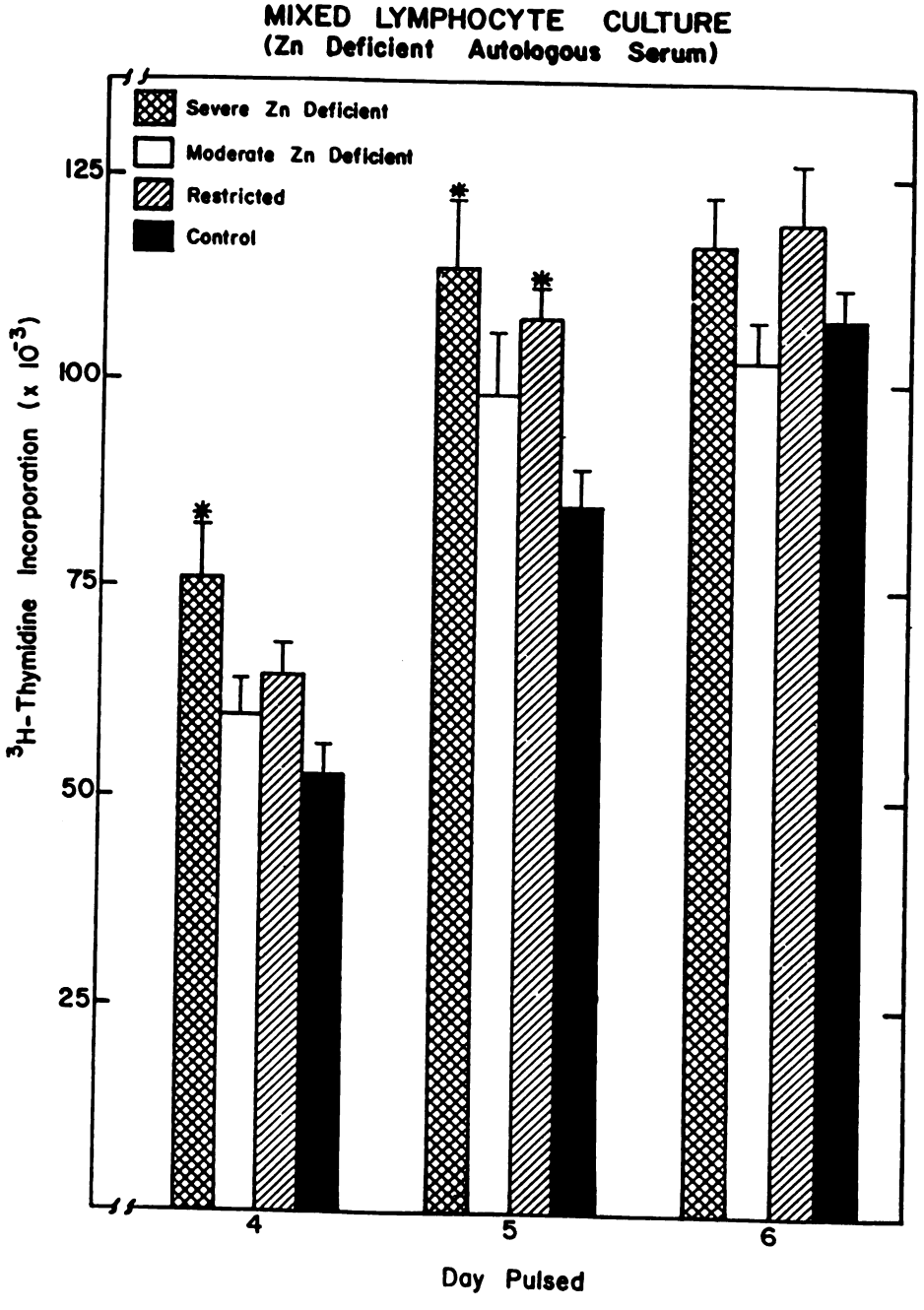

MIXED LYMPHOCYTE CULTURE
(Zn Deficient Autologous Serum)
Severe Zn Deficient
Moderate Zn Deficient
Restricted
Control
125
100
75
50
25
^{3}H-Thymidine Incorporation (x 10^{-3})
4
5
6
Day Pulsed

Figure 7. Kinetics of the MLC proliferative response in 0.5% zinc adequate autologous serum supplemented medium. A/J responder splenocytes (2.5×10^5) were cultured with 1×10^5 mitomycin C-treated C57Bl/6 splenocytes and pulsed for 18 hours with ^{3}H-thymidine on day 4, 5, or 6. Each bar represents the mean $\pm$ SEM for five or six mice. Asterisk indicates significance of $p < 0.05$ or better as compared to control responses.

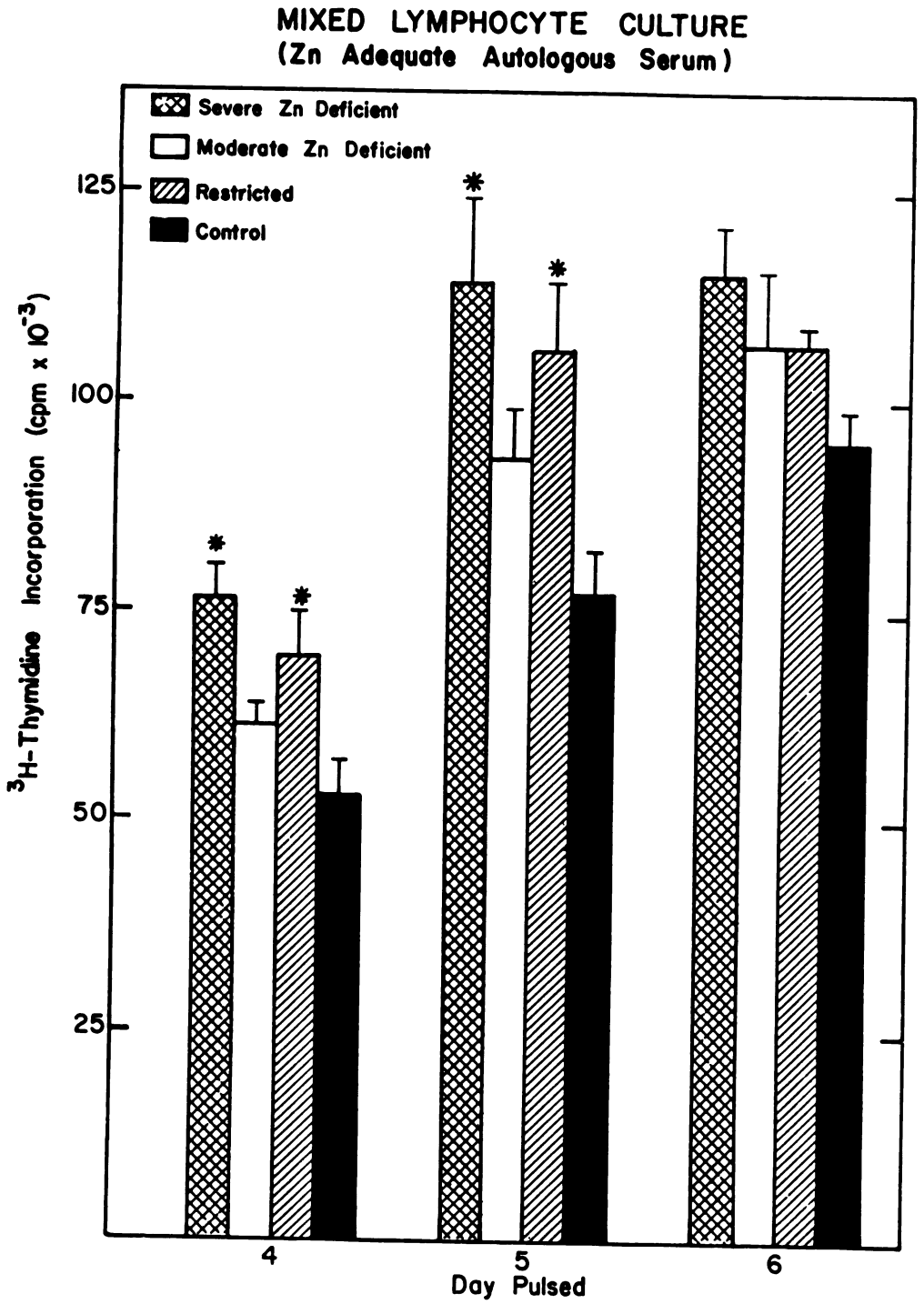

MIXED LYMPHOCYTE CULTURE
(Zn Adequate Autologous Serum)
Severe Zn Deficient
Moderate Zn Deficient
Restricted
Control
125
100
75
50
25
^{3}H-Thymidine Incorporation (cpm x 10^{-3})
4
5
6
Day Pulsed

Figure 8. Dose curve of the MLC proliferative response in 0.5% zinc deficient autologous serum supplemented medium. A/J responder splenocytes (2.5×10^5) from severely zinc deficient, moderately zinc deficient, restricted, or control mice were cultured with mitomycin C-treated C57Bl/6 splenocytes and pulsed for 18 hours with ^{3}H-thymidine on day 5. Each point represents the mean $\pm$ SEM for five or six mice. Asterisk indicates significance of $p < 0.05$ or better as compared to control responses.

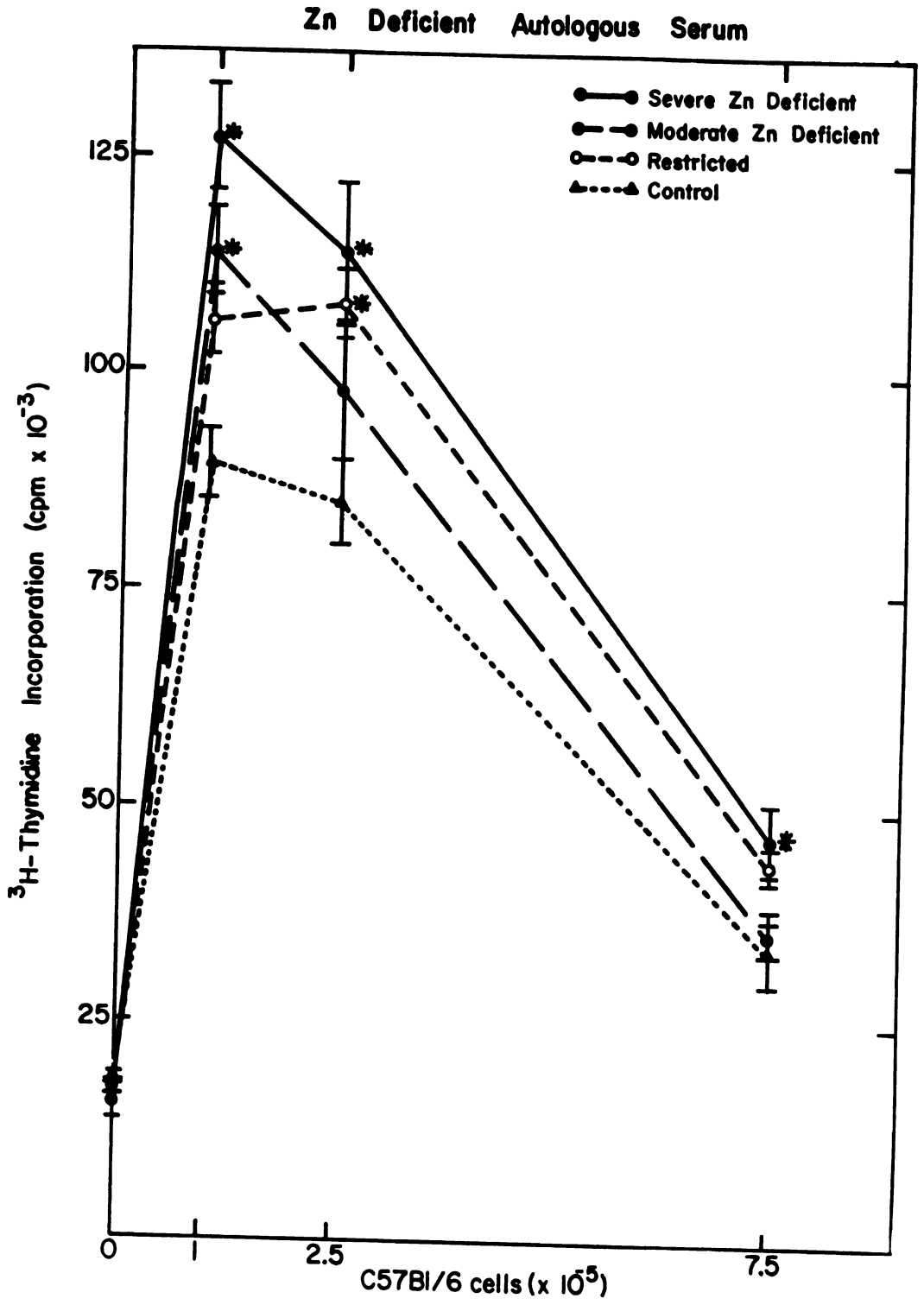
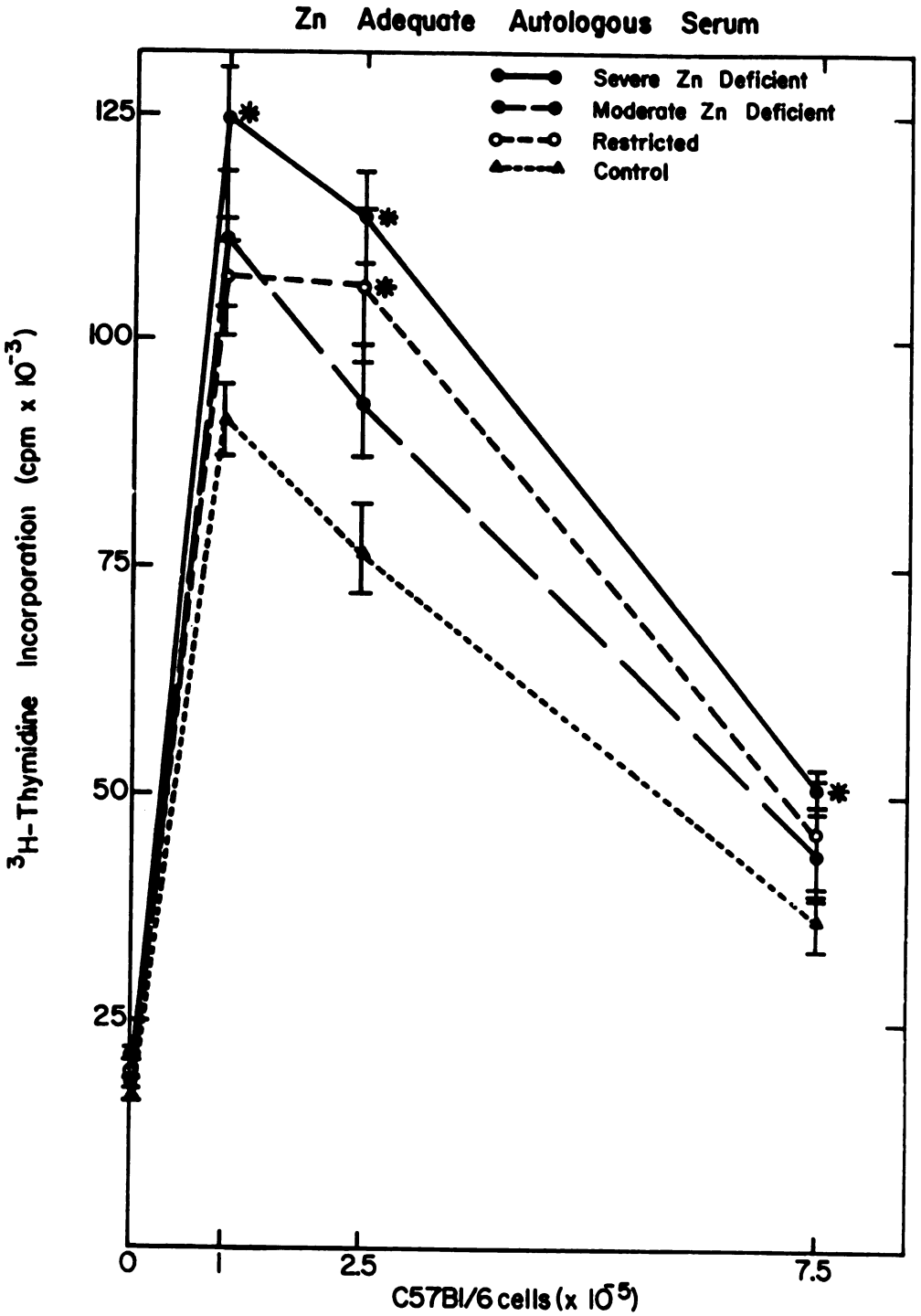

Zn Deficient Autologous Serum
Severe Zn Deficient
Moderate Zn Deficient
Restricted
Control
125
100
75
50
25
0
1
2.5
7.5
^{3}H-Thymidine Incorporation (cpm x 10^{-3})
C57Bl/6 cells (x 10^5)

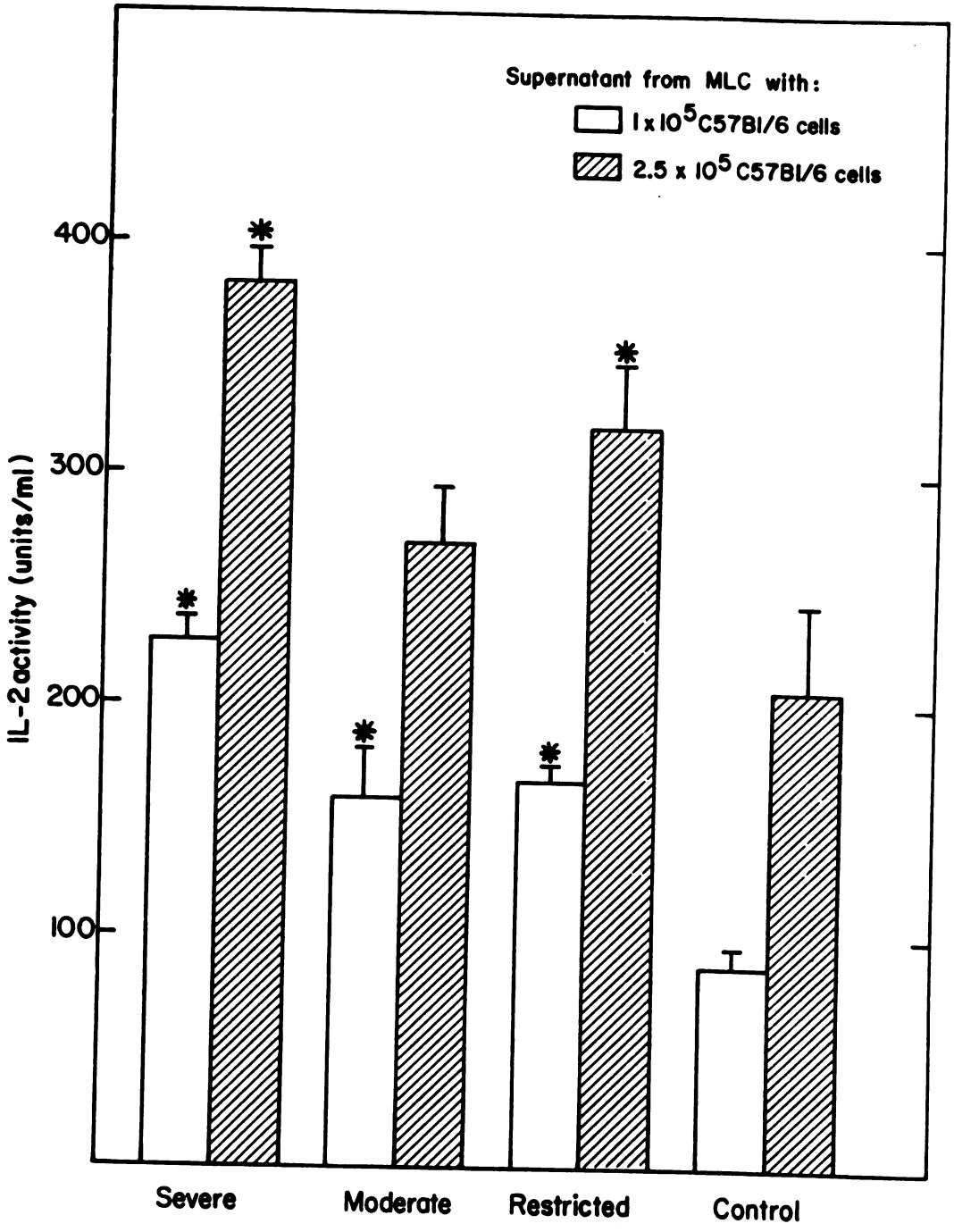
Figure 9. Dose curve of the MLC proliferative response in 0.5% zinc adequate autologous serum supplemented medium. A/J responder splenocytes (2.5×10^5) from severely zinc deficient, moderately zinc deficient, restricted, or control mice were cultured with mitomycin C-treated C57Bl/6 splenocytes and pulsed for 18 hours with ^{3}H-thymidine on day 5. Each point represents the mean $\pm$ SEM for five or six mice. Asterisk indicates significance of $p < 0.05$ or better as compared to control responses.


Zn Adequate Autologous Serum
Severe Zn Deficient
Moderate Zn Deficient
Restricted
Control
125
100
75
50
25
0
1
2.5
7.5
^{3}H-Thymidine Incorporation (cpm x 10^{-3})
C57Bl/6 cells (x 10^5)

8, 9). Regardless of whether the serum supplement contained adequate or limiting levels of zinc, proliferation by splenocytes from zinc deficient A/J mice was increased compared to proliferation by splenocytes from zinc adequate mice (Figures 8, 9). When zinc deficient autologous serum was used (40 μg Zn/dl), the severely and moderately deficient groups gave significantly increased proliferative responses of 141% and 127%, respectively (Figure 8), of the zinc adequate group. Using zinc adequate autologous serum (107 μg Zn/dl) (Figure 9), the severe and moderate groups again exhibited enhanced proliferative responses (137% and 122%, respectively, of controls). However, statistical analysis indicates that only the severe group was significantly different from control. In zinc adequate and deficient autologous serum, the proliferation by the restricted group was not significantly different from the control group at the optimal stimulatory condition of 1 x 10^5 C57Bl/6 target cells but was significantly different from controls at 2.5 x 10^5 C57Bl/6 target cells. Even though FCS supported greater overall proliferation than autologous serum, there was increased proliferation by the deficient groups regardless of the source of sera. Therefore, the level of zinc present in the sera does not appear to account for the enhanced proliferative response of splenocytes from the zinc deficient dietary groups.

Since proliferation by cells from zinc deficient mice was increased both in FCS and autologous serum, IL-2 activity was measured in supernatants from a MLC supplemented with FCS. Figure 10 shows that IL-2 activity was increased in the 48 hour supernatants from

Figure 10. IL-2 activity in 5% FCS supplemented medium harvested at 48 hours from quintriplet cultures of a MLC. A/J responder splenocytes (2.5×10^5) from severely zinc deficient, moderately zinc deficient, restricted, or control mice were cultured with 2.5×10^5 C57Bl/6 splenocytes. IL-2 activity was measured by the assay using the IL-2 dependent CTLL-2 cell line. Each bar represents the mean $\pm$ SEM for five or six mice. Asterisk indicates significance of $p < 0.05$ or better as compared to the respective control for each concentration of C57Bl/6 target cells.


Supernatant from MLC with:
1 x 10^5 C57Bl/6 cells
2.5 x 10^5 C57Bl/6 cells
IL-2 activity (units/ml)
400
300
200
100
Severe
Moderate
Restricted
Control

the severe, moderate, and restricted groups (185%, 131%, and 155% of controls, respectively) at optimal conditions with 2.5 x 10^5 C57Bl/6 target cells

The mitomycin C-treated cells serving as stimulator cells were unresponsive in the MLC. The mitomycin C treated C57Bl/6 cells did not proliferate in the presence of IL-2 nor did they respond to mitomycin C treated A/J splenocytes (Figure 11). Furthermore, IL-2 added to the culture containing only target cells was not absorbed nor was IL-2 activity produced by mitomycin C treated cells (Figure 12). Therefore, the proliferation and the production of IL-2 in the MLC was due only to the A/J splenocytes.

The observations that both IL-2 activity and cellular proliferation of splenocytes from the zinc deficient groups were elevated suggested that there might be a concommitant modulation of IL-2 receptor numbers. Therefore, it was of interest to examine the acquisition of IL-2 receptors during the MLC. The relative number of receptors was quantitated by indirect immunofluorescense of pooled cells from each dietary group on days 3, 4, 5, and 6 of a MLC supplemented with 5% FCS. On day 1 and 2 of the MLC the percentage of fluorescence was very close to background (data not shown). Cytofluorographic analysis of labelled cells from the four dietary groups showed that the number of receptors per cell was not altered by zinc deficiency since, for all groups, the mean channel number of fluorescence was approximately 555. However, the percentage of cells with IL-2 receptors was considerably increased for the severely zinc deficient group from day 3 (120% of controls) to day 6 (180% of

Figure 11. Proliferation of mitomycin C-treated splenocytes from mice fed normal laboratory chow. In the presence or absense of IL-2, mitomycin C-treated C57Bl/6 splenocytes pooled from six mice were incubated in 5% FCS supplemented medium with untreated or mitomycin C-treated A/J splenocytes from 3 mice for 5 days and pulsed for 18 hours with ^{3}H-thymidine. Each bar represents an individual sample.

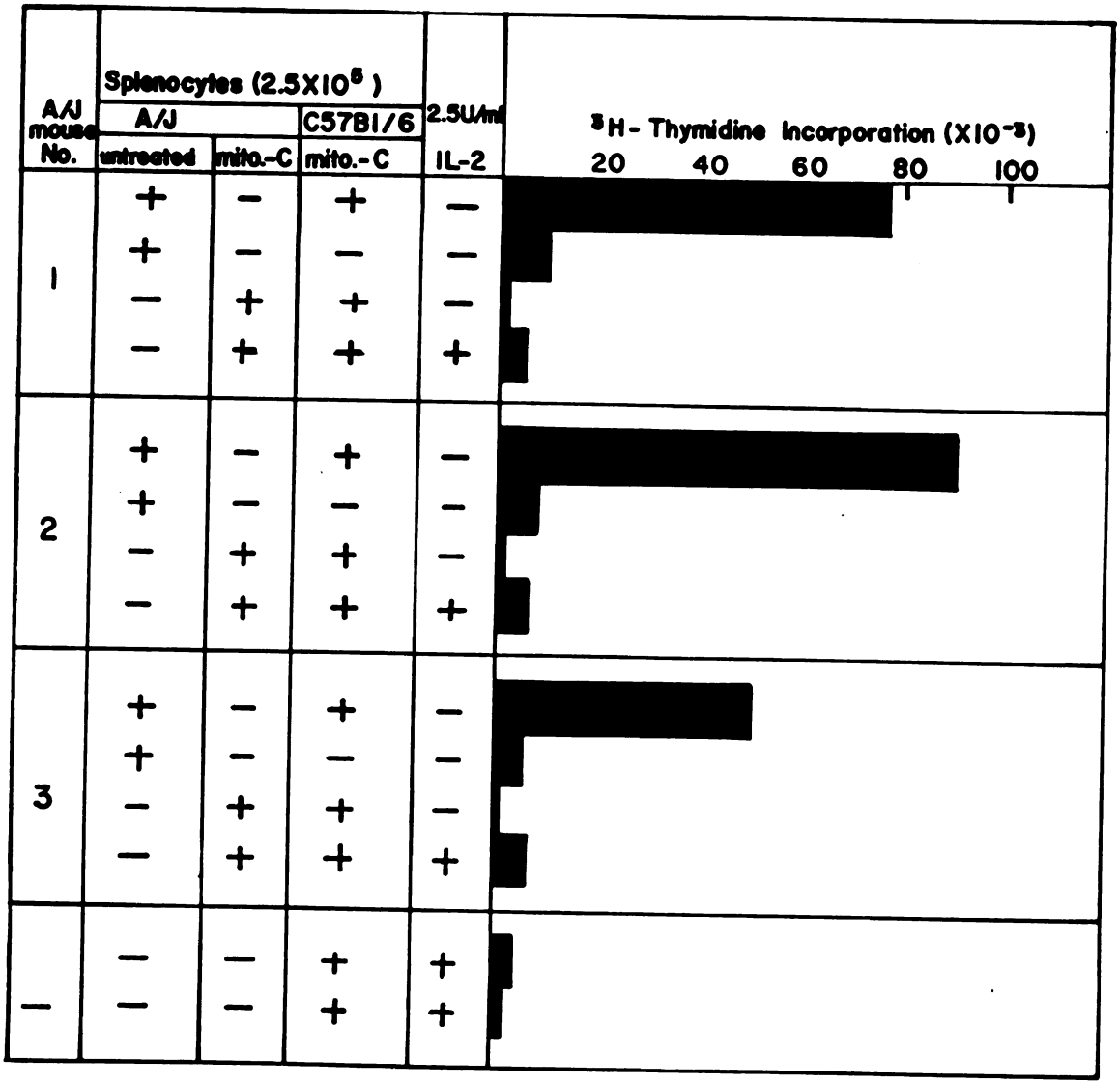

A/J mouse No.
Splenocytes (2.5X10⁵)
A/J
C57Bl/6
untreated
mito.-C
mito.-C
2.5U/ml IL-2
³H- Thymidine incorporation (X10⁻³)
20
40
60
80
100
1
2
3
—

Figure 12. IL-2 activity in medium harvested from mitomycin C-treated splenocytes from mice fed normal laboratory chow. In the presence or absense of IL-2, mitomycin C treated C57Bl/6 splenocytes pooled from six mice were incubated in 5% FCS supplemented medium with untreated or mitomycin C-treated A/J splenocytes from 3 mice. IL-2 activity was measured by the assay using the IL-2 dependent CTLL-2 cell line. Each bar represents an individual sample.

A/J mouse No.	Splenocytes (2.5×10^6) A/J untreated	A/J mito-C	C57Bl/6 mito-C	2.5 U/ml IL-2	IL-2 activity (Units / ml) 1 2 3
1	+	−	+	−	
	−	+	+	−	
	−	+	+	+	
2	+	−	+	−	
	−	+	+	−	
	−	+	+	+	
3	+	−	+	−	
	−	+	+	−	
	−	+	+	+	
−	−	−	+	+	

controls) of the MLC (Figure 13). The percentage of fluorescently labelled splenocytes from the moderately zinc deficent group was the same as that of the zinc adequate group. There was a slight increase compared to controls in the number of splenocytes in the restricted group with IL-2 receptors; however, this increase occurred in the final days of the MLC whereas the percentage of splenocytes with IL-2 receptors from the severely zinc deficient group was increased in the early stages of the MLC. In summary, allogeneic cell-stimulated proliferation and IL-2 production by the residual splenocytes from zinc deficient mice were increased compared to that for splenocytes from control mice. There was also an increase in the number of splenocytes with IL-2 receptors in the zinc deficient group; however, the number of IL-2 receptors per cell was the same for all groups.

Next, the functional capacity of the residual B-cells of the deficient mice was assessed by examining the ability of antigen-activated B-cells to produce immunoglobulins in response to SRBC. Splenocytes were removed from mice injected 5 days earlier with SRBC and analyzed for the numbers of cells producing antibody (plaque forming cells, PFC) using the Jerne plaque assay and the amount of antibody produced per PFC using a radioimmunoassay. The level of zinc introduced <u>in vitro</u> was minimized since the cells were incubated in medium supplemented with 1% BSA (0.5 μg Zn/dl). As in several previous studies (5, 7, 8, 14, 19, 21), the number of PFC per spleen was reduced to 50% in the deficient groups whereas the number of PFC per million splenocytes remained unaltered in the zinc deficient groups (data not shown). Table 2 shows that the average amount of

Figure 13. Percentage of splenocytes with IL-2 receptors. A/J responder splenocytes (2.5×10^5) from severely zinc deficient, moderately zinc deficient, restricted, or control mice were cultured with 7.5×10^5 C57Bl/6 splencytes in 5% FCS. On day 3, 4, 5, and 6, the IL-2 receptors on these splenocytes were labelled by indirect immunofluorescense. Each bar represents the percent of cells, excluding cellular debris, that were fluorescently labelled for pooled cells of five or six mice.

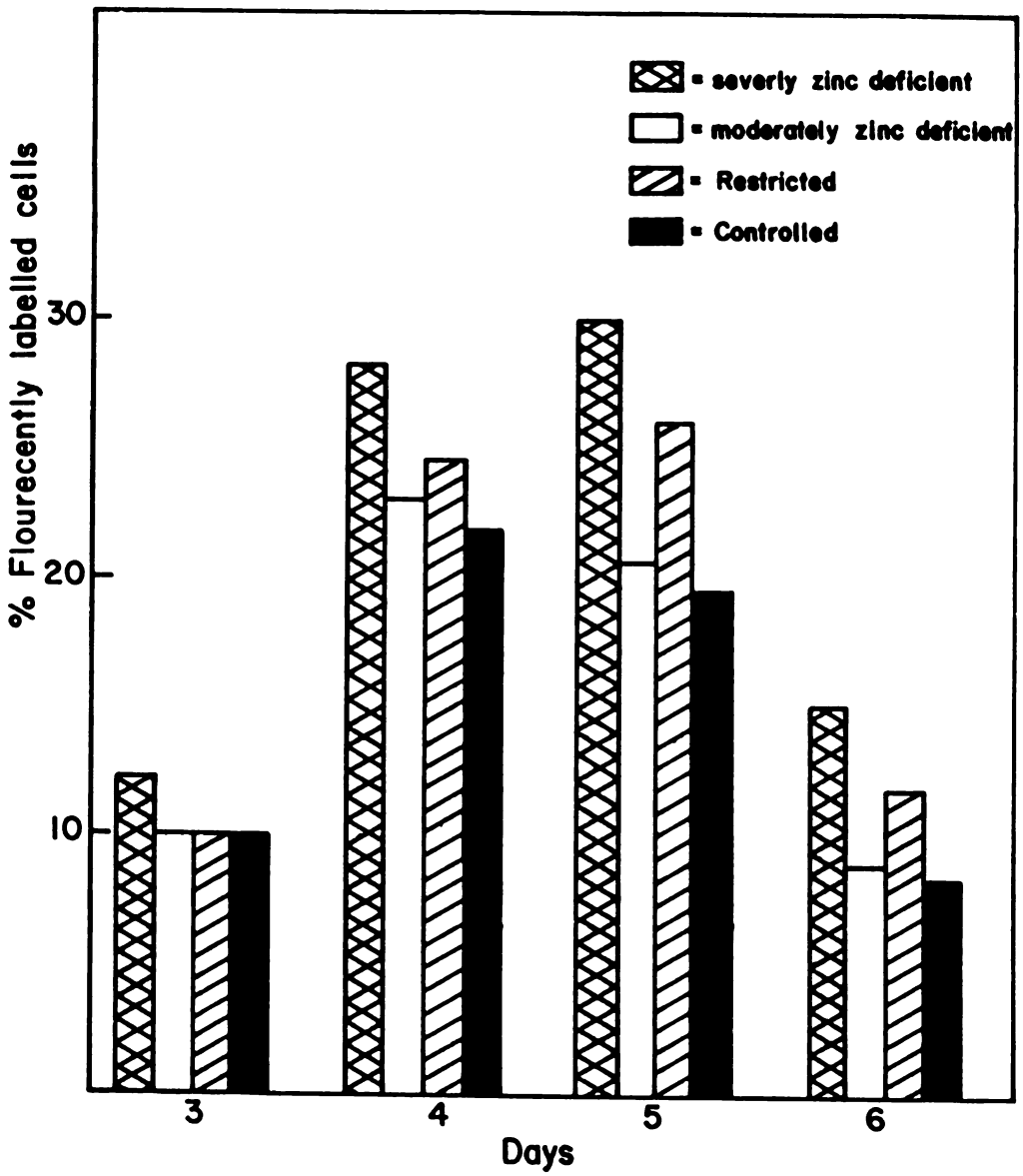

= severly zinc deficient
= moderately zinc deficient
= Restricted
= Controlled
% Flourecently labelled cells
30
20
10
3
4
5
6
Days

TABLE 2

Immunoglobulin Production/PFC

Dietary Groups	pg IgM/IgM PFC	pg IgG/IgG PFC
Severely Zinc Deficient	22.1 ± 4.7[a]	15.5 ± 3.1
Moderately Zinc Deficient	29.6 ± 5.2	6.9 ± 2.8
Restricted	18.3 ± 3.5	17.0 ± 5.8
Zinc Adequate	25.4 ± 2.2	12.9 ± 3.4
Unprimed Mouse	N.D.[b]	N.D.

[a] Mean ± SEM of 4 to 6 mice

[b] Not detectable

IgM produced per IgM PFC and the average amount of IgG production per IgG PFC for the zinc deficient and restricted groups is not significantly different than those values for the control group. Thus, the proportion of residual B-cells from zinc deficient mice which respond to sheep red blood cells and the amount of antibody produced by antibody secreting cells was normal.

DISCUSSION

Although a variety of immune responses are decreased in the zinc deficient animal (5, 7, 8, 9, 10, 11, 12, 13), the data presented in this chapter indicates that the residual lymphocytes from zinc deficient mice function as well as or better than lymphocytes from control mice. In response to Con A, splenocytes from either severely or moderately zinc deficient mice have the same response kinetics, proliferative capacity and ability to produce IL-2 as splenocytes from adequately fed mice under serum free conditions. This was so even though serum free conditions were used to alleviate the possibility of repair due to the addition of serum zinc. Exemplifing the importance for careful regulation of the addition of the deficient nutrient in in vitro studies, are other studies which have shown that microbicidal defects of macrophages from zinc deficient mice were rapidly reversed by the addition of exogenous zinc in vitro (22, 23).

Contradictory results are reported in the literature for stimulation of zinc deficient splenocytes by Con A (24, 25, 26, 28). Differences in experimental approaches account for most of the differences in results. Flynn (24) and Zanzonico et. al. (25) who utilized in vitro depletion of zinc by chelation found decreased proliferative splenocyte responses to Con A by splenocytes from deficient mice. Flynn (24) used chelex 100 for depletion of zinc from media to be used in his assays whereas Zanzonico et. al. (25) added EDTA directly to the stimulated splenocytes. The EDTA would chelate a lot of

other metals since it has a low affinity for zinc making comparisons between studies difficult. Indeed in those studies, there was no evidence that zinc was, in fact, inaccessible to the cells. With regard to Flynn's studies, it may be that short term in vitro depletion of zinc simply does not have the same effects as 30 days of zinc deprivation in vivo, especially, since in vivo zinc depeletion leads to activation of the stress axis (27) that adversely effects lymphocyte function. Studies by Gross et. al. (26) using zinc deficient rats showed that the Con A response was reduced for splenocytes but normal for thymocytes. However, there has been some controversy over this data since proliferation was presented as a stimulation index instead of counts per minute of total radioactivity. Data presented as stimulation indices can be misleading unless the counts per minute of the unstimulated controls are the same. Kramer et. al. (28) found that Con A responses by splenocytes from zinc deficient rats were increased in 15% heat-inactivated fetal calf serum but normal in 2% autologous serum from zinc deficient, restricted, or zinc adequate rats. The latter agrees with our results for zinc deficient mice.

In contrast to the normal mitogenic responses to Con A, proliferation and IL-2 production by T_H-cells in response to allogeneic cells was increased in both the severely and moderately zinc deficient groups; values for the severely zinc deficient group were most elevated. The increased response was not the result of repair by the deficient cells through utilization of zinc present in FCS since there was also an increased response in the presence of zinc deficient

autologous serum. The increased response to allogeneic cells may have been due to an increase in the proportion of cells with IL-2 receptors and/or to an increase in number of IL-2 receptors per cell during the onset of the MLC response. An increase in number of receptors per cell may lower the threshold for stimulation needed to begin cell-cycle progression (29). The data showed that there was no measurable increase in number of IL-2 receptors per cell in the zinc deficient groups during the MLC repsonse since the mean channel number of fluorescence was the same for all dietary groups. In contrast, there was a modest but measurable increase in the numbers of cells with IL-2 receptors in the severely zinc deficient group by day 3 of the MLC.

There are several possible explanations for the enhanced MLC responses. There may be more immature T-cells with the surface markers Lyt 2,3 and high levels of Lyt 1 (this cell type is now identified by Lyt 2,3 and L3T4) in the zinc deficient mice, since this cell type is thought to be the major responders in the primary MLC (30). Indeed, Nash et. al. (31) noted an increase in the proportion of immature T-cells in the spleens of zinc deficient mice. Therefore, in the deficient group, more cells would be available to respond in the MLC with a concommitant increase in IL-2 production and IL-2 receptors as was observed. Thus, in contrast to Con A, which stimulates many T-cell subsets (32), the MLC stimulates a more narrow subset of T-cells. Therefore, with proportional changes in cell numbers amoung T-cell subsets, one would not expect alterations in polyclonal proliferation in response to Con A but would expect

increased proliferative levels in a MLC with an increase in number of cells in the responding T-cell subset. An alternative explanation for the enhanced MLC may be that there is loss of IL-2 inhibitor activity in the zinc deficient mice. It has been suggested that there is an inhibitor of IL-2 driven T-cell proliferation (33). Tadmori, Kant and Kamoun (34) also propose that an inhibitor protein controls IL-2 mRNA production. Perhaps zinc plays some role in the production or function of this inhibitor so that with loss of zinc there is a loss of inhibition and consequently overproduction of IL-2 followed by increased cellular proliferation. Conversely, the degree of production of the inhibitor may be altered by the presence of corticosterone produced during the course of the zinc deficiency. Perhaps, the T-cell subset responding in the MLC has lost IL-2 inhibitor activity. Another possibility is that some other mechanism of suppression, such as loss of suppressor cell activity towards the MLC response but not the Con A response, was also impaired. Although the T_H-cell responses to allogeneic cells were increased, the residual T_H-cells were able to function at least as well as the zinc adequate controls. Therefore, the reduced T-cell dependent responses in vivo (5, 7, 8, 9, 10, 11, 12, 13) may be due to the reduced numbers of cells (5, 6) since those residual T-cells responding to Con A or allogeneic cells retain normal functional capacity.

The residual B-cells from zinc deficient mice also retain normal capacity to produce Ig. With initiation of the immune response to SRBC in the host, normal quantities of antibodies were produced by individual activated B-cells from zinc deficient mice compared to

B-cells from control mice. In addition, the number of antibody secreting cells was normal per million splenocytes, but the number of antibody secreting cells per spleen was reduced (5, 7, 8, 14, 19, 21). Previous reports also show that proliferation by residual B-cells from zinc deficient mice is normal or increased in response to several mitogens (5). The mitogenic response to lipopolysaccharide and dextran sulfate is increased for zinc deficient mice whereas the mitogenic response to purified protein derivative is the same as the zinc adequate controls. It was suggested that the elevated responses were due to an increase in number of immature B-cells in zinc deficient mice since the relatively immature subset of B-cells responds to lipopolysaccharide and dextran sulfate (35, 36). The above cited results and the present findings indicate that the reduced B-cell responses by the zinc deficient mouse are most likely due to reduced numbers of cells and not reduced functional capacity of the residual B-cells.

In conclusion, the functional capacities of the residual T-cells and B-cells were normal for zinc deficient mice with regard to stimulation with Con A or allogeneic cells, and SRBC, respectively. This is the first diverse study of the function of residual lymphocytes in a nutritionally depleted animal where the in vitro exposure to the deficient nutrient was regulated. The data indicated that the surviving lymphocytes in zinc deficient mice are normal for many immune functions and do not show evidence of significant alteration in cell processes dependent on zinc.

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Chapter 3

IMPROVED CONDITIONS FOR MEASURING H_2O_2 PRODUCTION BY RESIDENT MACROPHAGES: MODIFICATIONS FOR USE WITH PARASITES

ABSTRACT

The assay conditions described by Pick and Mizel which utilizes phenol red to measure H_2O_2 production by neutrophils or elicited macrophages has been improved so that the amount of H_2O_2 produced by resident macrophages can now be measured. As opposed to the negligible amounts of H_2O_2 production (0-5 μM) previously obtained, phorbol 12-myristate 13-acetate (PMA) or opsonized zymosan but not zymosan alone readily stimulated H_2O_2 production (30 μM) by resident peritoneal macrophages using the modified conditions for the Pick assay. The modifications made for optimal H_2O_2 production included an increase in the concentration of resident peritoneal macrophages, addition of 0.5 mM $CaCl_2$ to the reaction solution and an incubation at 37°C under ambient air. Addition of 2 mM NaN_3 to inhibit scavenging of H_2O_2 by catalase did not affect H_2O_2 production by resident macrophages. When attempting to stimulate resident macrophages with the parasite Trypanosoma cruzi (T. cruzi), it was found that the phenol red employed as the substrate in the Pick assay rapidly killed the parasites. This problem was rectified by using an alternative substrate, homovanillic acid (HVA), and eliminating the NaN_3 from the reaction solution. With HVA as substrate, the optimal conditions for H_2O_2 production included the presence of 0.09 mM $CaCl_2$ and 0.05 mM $MgCl_2$ and incubation at 37°C with 7% CO_2 and ambient air. In the past it had not been possible to directly measure H_2O_2 production by macrophages stimulated with T. cruzi. With the modifications suggested herein, consistent and good production of H_2O_2 (3-10 μM)

could be obtained for the first time. Further, it was noted that opsonized T. cruzi elicited at least twice as much H_2O_2 production as nonopsonized parasites. In addition, as the number or ratio of parasites to macrophages increased, there was a corresponding increase in H_2O_2 production.

In summary, now it is possible to measure H_2O_2 production by resident macrophages stimulated with nonliving agents such as PMA or opsonized zymosan or with a living pathogen such as T. cruzi. Futhermore, the HVA assay will now make it possible to better assess the significance of H_2O_2 in the killing of T. cruzi by infected macrophages.

Introduction

An assay described by Pick et. al. (1) has been commonly used to measure H_2O_2 production by neutrophils and elicited macrophages (1-4). This assay utilizes the horseradish peroxidase catalyzed oxidation of phenol red to an unknown product that absorbs at 610 nm. It must be emphasized that in the past H_2O_2 production by resident macrophages has not been measurable since the sensitivity of this assay was far too low for quantitation of resident macrophage H_2O_2 production (2, 5, 6, 7, 8, 9). Conditions were described herein that made it possible to assess H_2O_2 production by resident macrophages.

However, for our studies, it was necessary to measure H_2O_2 production from resident macrophages using T. cruzi since we were interested in examining the functional capacity of resident macrophages from zinc deficient mice. These macrophages are deficient in their ability to associate with and kill the unicellular parasite, Trypanosoma cruzi (T. cruzi) (10, 11). Since destruction of T. cruzi has been assumed to be related to the amount of H_2O_2 produced (12, 13), we wanted to compare the ability of macrophages from zinc deficient mice and those from zinc adequate mice to produce H_2O_2. Defective H_2O_2 production was presumed to be one explanation for the reduced ability of macrophages from deficiency to kill parasites.

For this part of the studies, it became necessary to modify the prevailing assay conditions so that the amount of H_2O_2 produced by resident macrophages could be measured. This was done. However, it

was subsequently found that T. cruzi was killed by phenol red which is the major substrate of the Pick assay (1). This would explain why in the past other investigators were unable to use the parasite as the stimulant for H_2O_2 production. Instead, the ability of macrophages to produce H_2O_2 upon stimulation with the chemical PMA had been mathematically correlated with killing of T. cruzi by the macrophages (14). In addition, H_2O_2 production by infected cells had been demonstrated qualitatively by a cytochemical stain for H_2O_2 (12). Thus, it was presumed that H_2O_2 was essential to killing of T. cruzi since the ability of phagocytic cells to kill T. cruzi had been shown to be inhibited by the H_2O_2 scavenger, catalase, but not by inhibitors of other oxygen metabolites (13, 14).

The above experiments were the sole evidence that T. cruzi stimulated H_2O_2 production and that the H_2O_2 produced was essential to the killing of T. cruzi. Yet, it had never been shown directly that T. cruzi stimulated H_2O_2 production by macrophages. Therefore, additional modifications were made in the H_2O_2 assay using HVA as a substrate so that T. cruzi could be used as a stimulant of resident peritoneal macrophages. This modified assay will be important in determining the significance of H_2O_2 to the killing of T. cruzi. Likewise, it will also be possible to more clearly assess the role of H_2O_2 in the killing of other living pathogens and parasites such as Leshmania and Plasmodium falciparum.

MATERIALS AND METHODS

Animals. A/J female mice were purchased from Jackson Laboratory, Bar Harbor Maine. Female Crl-CD-1 (IRC)BR Swiss mice were purchased from Charles Rivers, Portage, Michigan.

Collection of mouse serum. Serum was collected from mice by severing the subclavian artery. The blood was incubated for 15 minutes at 37°C followed by several hours at 4°C. Mouse serum was complement inactivated by incubating at 56°C for 30 minutes.

Isolation of Trypanosoma cruzi (T. cruzi). Four week old Crl-CD-1 (IRC)BR Swiss female mice were infected intraperitoneally with 2×10^5 blood forms (trypomastigotes) of the Tulahuén strain of T. cruzi. Twelve to 14 days later, blood was collected from the axillary artery of mice anesthetized with ether. Blood was collected in tubes containing heparin or disodium ethylene-diaminotetracetate (EDTA) powder so that the final concentrations were 25 U/ml or 2.5 mg/ml, respectively. Parasites from the blood were separated on a lympholite gradient (isolymph, Gallard-Schlesinger, Carle Place, N.Y.) (15) and passed through a diethylaminoethylcellulose column (16). The parasites were centrifuged (800 x G, 15 minutes, 4°C) and resuspended in Dulbecco's modified minimal essential medium (DMEM, Gibco, Grand Island, NY) supplemented with 100 IU penicillin and 100 µg streptomycin / ml. One hundred percent of the parasites were viable and in the trypomastigote form as determined by morphology and motility. Before use in the assays for H_2O_2, the parasites were

washed two times in phosphate buffered saline (PBS) with 1% glucose and resuspended in the reaction solution for the H_2O_2 assay.

Preparation of opsonized zymosan and opsonized T. cruzi. One mg of zymosan, a yeast cell extract (Sigma, St. Louis, MO), was incubated with one milliliter of complement inactivated normal mouse serum from A/J mice for 1 hour at 37°C. The opsonized zymosan was washed 2 times and resuspended in 10 mM PBS (pH 7.4). T. cruzi trypomastigotes were opsonized by incubation for 1 hour at 37°C in 10 mM PBS (pH 7.4) with 1% glucose and 20% complement inactivated serum from chronic T. cruzi infected CD1 mice. The opsonized T. cruzi were washed 2 times and resuspended in 10 mM PBS (pH 7.4). The serum was obtained during the chronic phase of experimental Chagas' disease which occurs 5-6 weeks after the initial infection. Chronic mice have normal immune responsiveness but continue to harbor the parasite and produce T. cruzi-specific antibodies (17, 18).

Collection, isolation, and identification of peritoneal macrophages. Cells were harvested from the unelicited mouse peritoneum by lavage with 5 ml of cold (4°C) DMEM and 0.5% w/v gamma globulin free bovine serum albumin (BSA, Calbiochem, La Jolla, CA) (19). Cells were adhered to tissue culture plates for 1 1/2 hours at 37°C and 7% CO_2-ambient air. Nonadherent cells were removed by washing with 37°C DMEM. The percentage of macrophages/monocytes adhered to the culture plate was determined by staining for nonspecific esterase activity as detailed in the modification (20) of the technique described by Yam et. al. (21). Always, 94-100% of the adhered cells

were identified as monocytes/macrophages by staining for nonspecific esterase and by morphology.

Phenol Red Assay. The assay conditions described by Pick and Mizel (1) were modified for use with resident macrophages. One hundred microliters of 7 x 10^6 resident peritoneal exudate cells/ml were added per well to a 96 well flat bottom plate (Corning Glass Works, Corning, NY) and allowed to adhere for 1 1/2 hours. After removing nonadherent cells, the adherent macrophages were washed with 10 mM phosphate buffered saline (PBS) - 1% glucose (37°C). Forty microliters of the assay solution consisting of 140 mM NaCl, 0.5 mM $CaCl_2$, 10 mM potassium phosphate buffer (pH 7.4), 2 mM NaN_3, 5.5 mM glucose were supplemented with 0.56 mM phenol red (United States Biochemical Corporation, Cleveland, Ohio), and 19 U/ml horseradish peroxidase (Sigma, St. Louis, MO) and added to each well. NaN_3 was included since it inhibits catalase, the cytoplasmic scavenger of H_2O_2. Phorbol 12-myristate 13-acetate (PMA, Consolidated Chemicals) was diluted in ethanol and added to the cells in a volume of 2 µl per well so that the ethanol concentration was less than 1%. Opsonized zymosan or PMA stimulated cells were incubated for 90 minutes at 37°C under a humidified atmosphere of 7% CO_2-ambient air, ambient air, or 10% CO_2, 7% O_2 and 83% N_2. Standards consisted of wells containing reaction solution and 0-60 µM H_2O_2 but no cells. Nonspecific production of H_2O_2 was determined by including wells containing cells but no stimulant or stimulant but no cells. The reactions were stopped and protein dissolved by the addition of 2 µl of 10 N NaOH. The absorbance at 610 nm of pooled triplicates was

determined using a microcuvette and a spectrophotometer (Gilford, Oberlin, Ohio). Macrophage protein was determined by the Lowry (22). The data are presented as nmoles H_2O_2/mg macrophage protein from the following calculation: (μM H_2O_2 derived from the standard curve) X (1 liter/1000 ml) X (0.132 ml final volume of sample) X (1000 nmoles/1 μmole)/(mg macrophage protein determined by the Lowry) = nmoles of H_2O_2/mg macrophage protein.

Homovanillic acid assay for H_2O_2. The assay conditions described by Ruch et. al. (23) were modified for use with resident macrophages with T. cruzi trypomastigotes as the stimulant. One milliliter of 2 X 10^6 resident peritoneal cells/ml was added per well to a 24 well flat bottom plate (Corning) and allowed to adhere for 1 1/2 hours. After removal of the nonadherent cells, the adherent macrophages were washed with PBS - 1% glucose (37°C). Two hundred microliters of reaction solution which consisted of PBS, 1% glucose, 0.09 mM $CaCl_2$, 0.05 mM $MgCl_2$, 200 μM homovanillic acid, and 2 U/ml horseradish peroxidase were added per well. The opsonized zymosan or PMA was added in a small volume (2-10 μl). When parasites were the stimulant, they were resuspended in the 200 ul of reaction solution immediately prior to addition to the macrophages. Standards consisted of wells containing reaction solution and 0-20 μM H_2O_2 but no cells. Non-specific production of H_2O_2 was determined by including wells containing cells but no stimulant or stimulant but no cells. The plates were centrifuged at 50 x G for 3-4 minutes to increase parasite-macrophage interactions and incubated for 90 minutes in a humidified atmosphere at 37°C and 7% CO_2-ambient air. The reaction was stopped

by the addition of 25 µl of 25 mM EDTA and 0.1 M glycine at pH 12. The relative fluorescence of the homovanillic acid dimer was determined using a spectrofluorometer (Perken-Elmer 650-40) equipped with a microcuvette. The excitation and emission wavelengths were 312 nm and 420 nm, respectively. The slit widths for excitation and emission were 2 nm and 5 nm, respectively. The amount of H_2O_2 in the samples was analyzed by linear regression of the standard curve. The amount of macrophage protein was determined by the Lowry (22). The data are presented as nmoles H_2O_2 / mg macrophage protein from the following calculation: (µM H_2O_2 derived from the standard curve) X (1 liter/1000 ml) X (0.225 ml final volume of sample) X (1000 nmoles/1 µmole) / (mg macrophage protein as determined by the Lowry) = nmoles H_2O_2 / mg macrophage protein.

Association of T. cruzi with macrophages. After incubating the macrophages with parasites for 90 minutes in DMEM at 37°C and 7% CO_2-ambient air, the nonassociated parasites were removed by washing three times with PBS - 1% glucose. The cultures were incubated in DMEM for another 18 hours and/or fixed with methanol for 5 minutes, allowed to dry, and stained for 1 hour with Giemsa in 10 mM phosphate buffer (pH 6.8). The cells were washed one time with 10 mM phosphate buffer (pH 6.8). Numbers of parasites per 100 macrophages and percent of macrophages associated with parasites were determined by counting at least 200 cells per replicate.

Macrophage viability. Viability of the adherent macrophages was determined using the trypan blue exclusion method. Macrophage viability was always >95%.

Statistics. The mean and standard error of the mean were calculated for each group. Probability values were determined by a completely random Anova followed by Dunnett's t Test.

RESULTS

The standard assay for H_2O_2 described by Pick and Mizel (1) utilizes the horseradish peroxidase catalyzed reduction of phenol red to an unknown derivative absorbing at 610 nm. In the past, resident macrophages (i.e. macrophages not activated or elicited *in vivo*) have been shown to produce very low or nonmeasurable amounts of H_2O_2 even though a variety of stimulants were tested (2, 5, 6, 7, 8, 9). Therefore, to improve the assay conditions for use with resident macrophages, the concentration of macrophages was increased from the usual range of 3×10^5 peritoneal cells plated/ml reaction solution to 1×10^7 peritoneal cells plated/ml of reaction solution (1) to 1.75×10^7 peritoneal cells plated/ml reaction solution. Thus, the concentration of macrophages herein was 2 to 58 times that described in Pick and Mizel (1).

Using these concentrations of cells, it became possible to readily measure peroxide production by resident macrophages using a variety of probes (Table 1). With the standard assay, 20 μg of PMA elicited the production of 10 μmoles of H_2O_2 per mg macrophage protein which was the optimum that could be obtained under these conditions. However, as little as 12.5 μg of PMA elicited four times as much peroxide production using the suggested cell concentrations (Table 1). More striking differences were obtained with opsonized zymosan which must be phagocytosed by the macrophage. Using the modified conditions, a range of concentrations of opsonized zymposan failed to produce detectable quantities of H_2O_2 (approximately 1.0 nmoles

Table 1

H_2O_2 Production by Resident Peritoneal Macrophages

Assay Conditions	Stimulant	Amount of Stimulant	nmoles H_2O_2/mg macrophage protein
Pick & Mizel Phenol Red Assay[a]	---	0	3.5 ± 1.7
	PMA	2 ng	3.4 ± 0.6
		20 ng	10.0 ± 2.4
		80 ng	8.8 ± 1.3
	opsonized zymosan	20 µg	1.1 ± 7.2
		50 µg	0.0 ± 4.8
		100 µg	1.4 ± 5.7
Modified Phenol Red Assay[b]	---	0	7.0 ± 1.3
	PMA	0.0125 ng	8.8
		12.5 ng	40.5 ± 2.9
		250 ng	40.4 ± 1.0
		500 ng	43.6 ± 3.9
	opsonized zymosan	12 µg	42.6 ± 4.0

[a] Assay described in Pick, E., and Mizel, D. (1981) J. Immunol. Methods 46, 211-226. Conditions for comparison: 0.3×10^5 to 10×10^5 peritoneal cells in 100 µl reaction solution, no $CaCl_2$, no NaN_3, incubation in 5% CO_2-ambient air at 37°C.

[b] Modified Phenol Red Assay described herein. Conditions for comparison: 21×10^5 peritoneal cells in 120 µl reaction solution, 0.5 mM $CaCl_2$, 2 mM NaN_3, incubation in ambient air at 37°C.

H_2O_2/mg macrophage protein) (Table 1). However, 40 μmoles H_2O_2/mg macrophage protein were readily obtained with the modified assay system. It should be noted that the quantities of H_2O_2 produced by both PMA and zymosan were readily and reliably quantitated since they coincided with the middle of the standard curve (Figure 1).

Since previous investigators had used a variety of atmospheric conditions with mononuclear cells (1-14), various incubation conditions were also evaluated for the modified phenol red assay. The conditions tested were ambient air, 7% CO_2-ambient air, and the conditions described by Mishell and Dutton for leukocyte cultures that uses (24) 10% CO_2, 7% O_2 and 83% N_2. Under ambient air (about 20% O_2 and <0.1% CO_2) or 7% CO_2-ambient air, there was twice as much H_2O_2 production (Figure 2) by resident macrophages whether opsonized zymosan or PMA were used than when 10% CO_2, 7% O_2, and 83% N_2 was used. Thus, an increase in atmospheric O_2 caused a significant increase in H_2O_2 production (Figure 2) when utilizing the phenol red assay. This is probably due to the presence of more O_2 as a substrate for NADPH oxidase which catalizes the production of O_2^- which in turn dismutates to H_2O_2. Therefore, the remaining studies using the phenol red assay for H_2O_2 were carried out under ambient air.

In an attempt to optimize the amount of H_2O_2 produced by the resident macrophages, $CaCl_2$ (0.5 mM) which is required for phagocytosis and NaN_3 (2 mM) which inhibits the H_2O_2-scavenger catalase were added to the reaction solution (Figure 3). When present, calcium increased the amount of H_2O_2 production (525%) when macrophages were stimulated with opsonized zymosan, a particle which is phagocytosed

Figure 1. Standared curve for H_2O_2 production in the modified phenol red assay. Various concentrations of reagent H_2O_2 was incubated with the reaction solution for one hour since the reaction goes to completion by 1 hour and the product is stable for several hours.

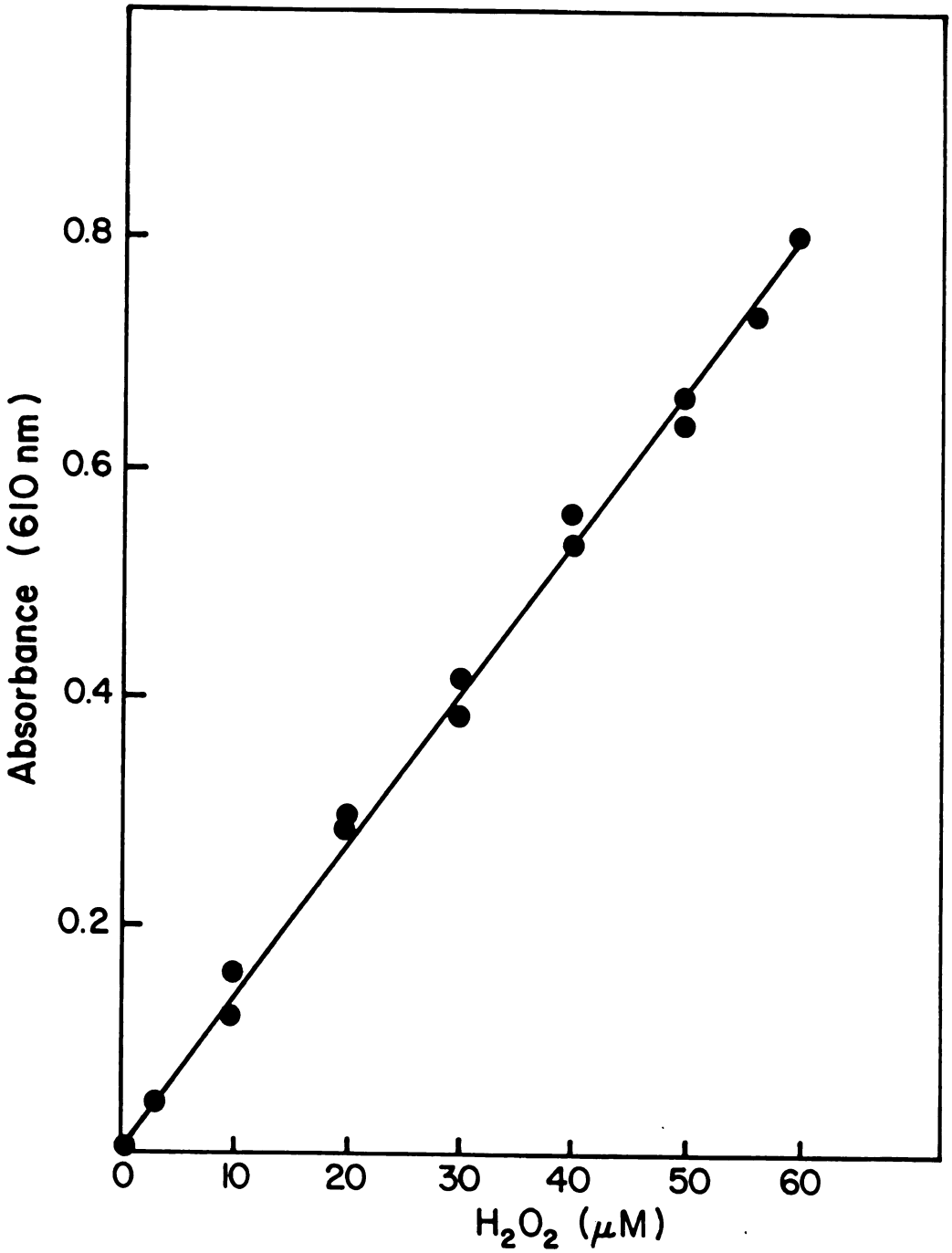

Absorbance (610 nm)
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
H_2O_2 (μM)

Figure 2. Modified phenol red assay: Atmospheric conditions for optimal H_2O_2 production by adherent macrophages from 2.1 x 10^6 resident peritoneal cells. Macrophages were incubated with 12 μg opsonized zymosan or 3 μg PMA for 2 hours in the presence of 0.5 mM $CaCl_2$ and 2 mM NaN_3.

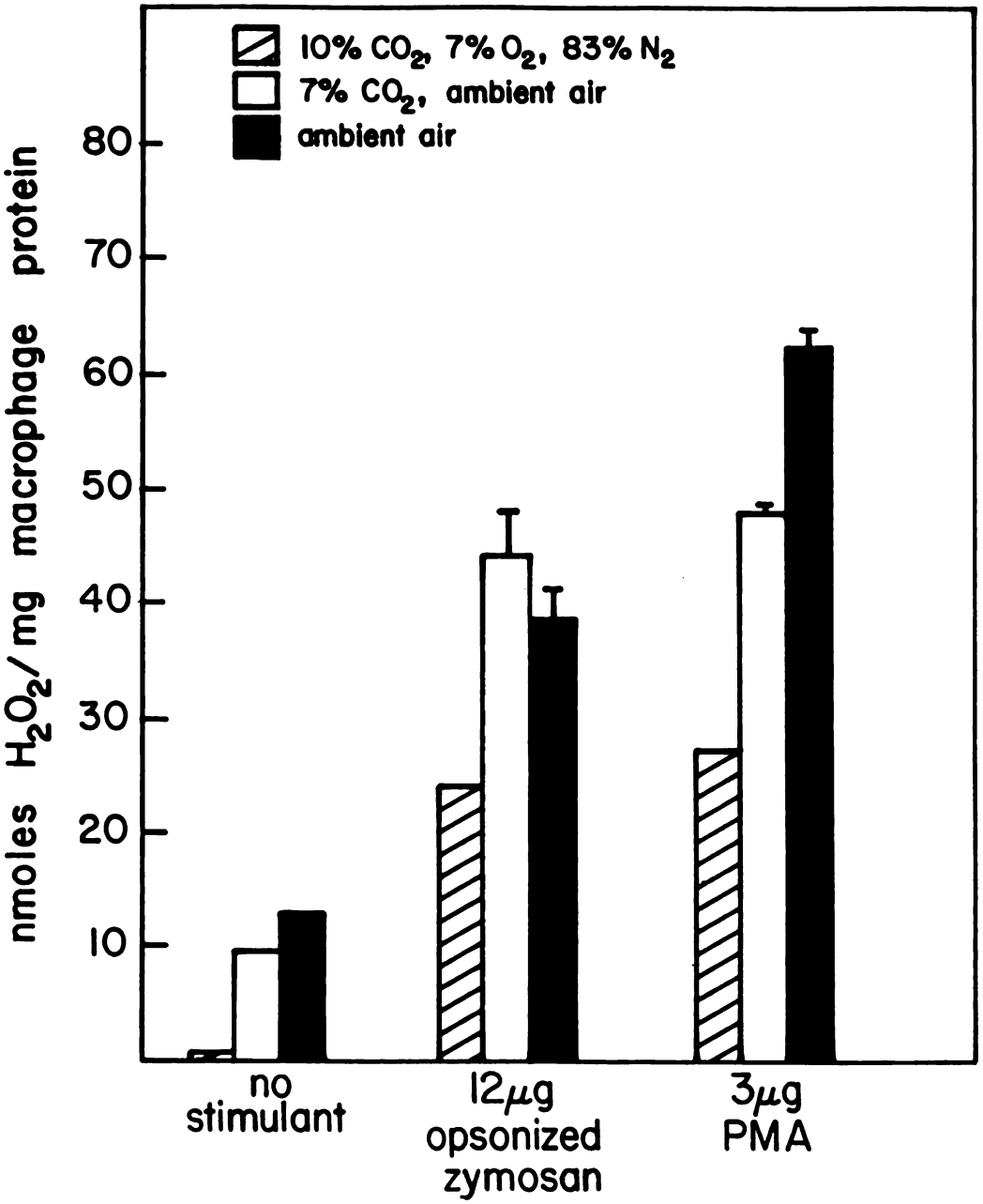
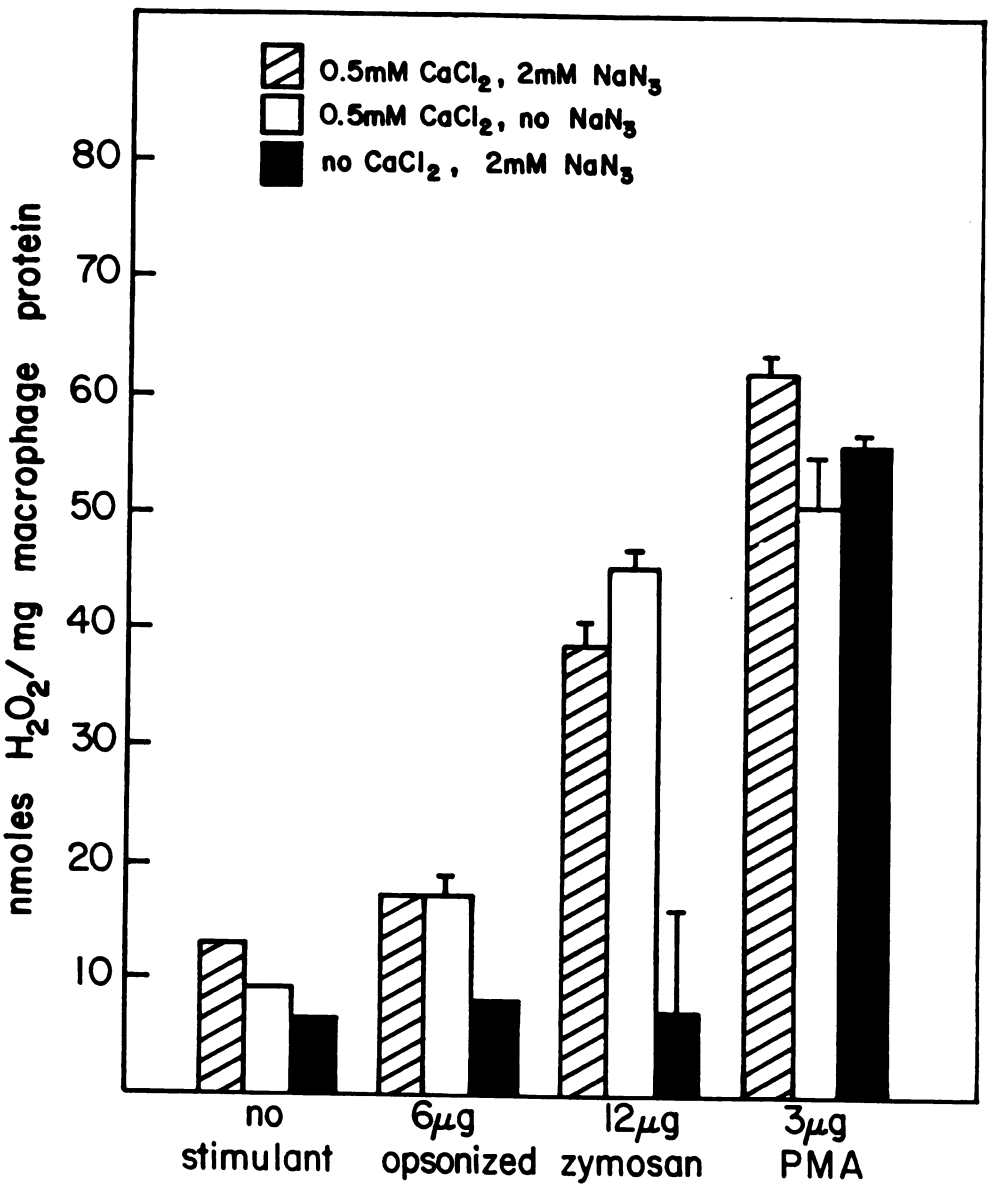

10% CO2, 7% O2, 83% N2
7% CO2, ambient air
ambient air
nmoles H2O2/mg macrophage protein
80
70
60
50
40
30
20
10
no stimulant
12μg opsonized zymosan
3μg PMA

Figure 3. Modified phenol red assay: Requirements for 0.5 mM $CaCl_2$ and 2 mM NaN_3 for optimal H_2O_2 production by adherent macrophages from 2.1 x 10^6 resident peritoneal cells. Macrophages were incubated with 6 or 12 μg opsonized zymosan or 3 μg PMA for 2 hours under ambient air.

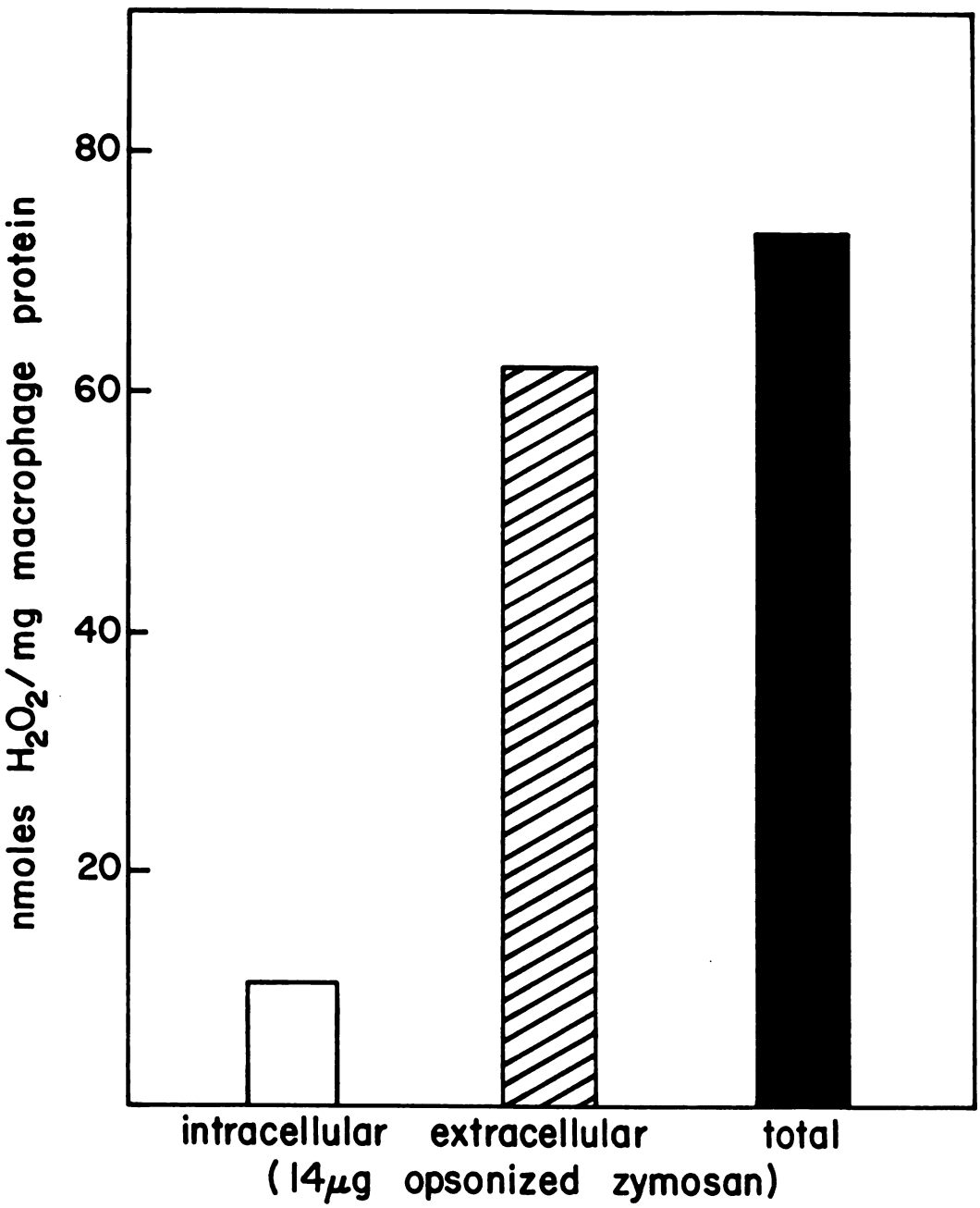

0.5mM CaCl2, 2mM NaN3
0.5mM CaCl2, no NaN3
no CaCl2, 2mM NaN3
nmoles H2O2/mg macrophage protein
80
70
60
50
40
30
20
10
no stimulant
6μg opsonized zymosan
12μg
3μg PMA

upon binding to receptors on macrophages (Figure 3). Addition of calcium did not significantly increase H_2O_2 production (7%) when the stimulant was the molecule PMA. This is probably due to the fact that PMA diffuses through the macrophage membrane; thus, receptor mediated phagocytosis does not occur (Figure 3). The concentration of NaN_3 usually used to inhibit cellular catalase (25-32) did not affect the amount of H_2O_2 produced by the macrophages stimulated with PMA or opsonized zymosan. This suggests that cytoplasmic catalase is not scavenging significant amounts of H_2O_2. In summary, under the optimal conditions described above, H_2O_2 production by resident macrophages can be measured since the amount of H_2O_2 produced is now near the middle of the linear portion of the standard curve.

To determine the distribution of H_2O_2 production, extracellular, intracellular, and total amounts of H_2O_2 were measured. The total amount of H_2O_2 produced by the macrophages can be assessed by adding NaOH directly to the cells and reaction solution. Extracellular H_2O_2 may be measured by adding the NaOH to the reaction solution after removal from the cell monolayer. Fresh reaction solution and NaOH was added to the remaining cell monolayer to determine the amount of intracellular H_2O_2. As can be seen from Figure 4, the majority of detectable H_2O_2 was in the extracellular environment with negligible amounts remaining in the intracellular environment.

Although this modified phenol red assay can be used with nonliving stimulants, when the stimulant is a living parasite such as the obligate intracellular parasite T. cruzi, no H_2O_2 was produced. It was noted that the parasites died rapidly in the reaction solution.

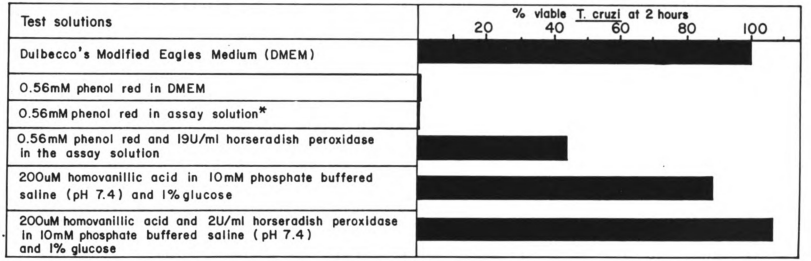
Figure 4. Modified phenol red assay: Measurement of total, extracellular, and intracellular H_2O_2 produced by adherent macrophages from 2.1 x 10^6 resident peritoneal cells. Macrophages were incubated with 14 μg opsonized zymosan in 0.5 mM $CaCl_2$, and 2 mM NaN_3 under ambient air. Total H_2O_2 was measured by adding NaOH to stop the reactions to the cells and reaction solution. Extracellular H_2O_2 was measured by adding the NaOH to the reaction solution after removal from the cells. Intracellular H_2O_2 was measured by adding fresh reaction solution to the remaining adherent macrophages and adding NaOH.


80
60
40
20
nmoles H_2O_2/mg macrophage protein
intracellular
extracellular
total
(14μg opsonized zymosan)

Analysis of the components of the reaction solution indicated that phenol red, the substrate for the assay, was toxic to T. cruzi (Figure 5). The toxicity of phenol red was tested by incubating the parasites at 7% CO_2-ambient air for 2 hours in several solutions (without macrophages). Parasites were 100% viable in DMEM. Upon addition of 0.56 mM phenol red to either DMEM or the assay solution, no viable parasites remained after 2 hours. Interestingly, if horseradish peroxidase was added to the normal reaction solution at the same time as phenol red, it provided some protection to the parasites (approximately 40% viability). The concentration of phenol red can not be reduced in the assay since the substrate would becoming limiting in the reaction (1). An alternative substrate for the assay, homovanillic acid (HVA), has been described by Ruch et. al. (23). HVA has been rarely used as a substrate when assaying for cellular production of H_2O_2. However, HVA proved very useful in our system since the HVA concentration (200μM) necessary for measuring H_2O_2 production did not destroy T. cruzi either in the presence or absence of horseradish peroxidase in the reaction solution (Figure 5).

As in the case for phenol red, it was necessary to modify the conditions of the HVA assay described by Ruch et. al. (23) for use with resident peritoneal cells. The concentration of cells was increased from 2.5 x 10^5 cells plated/ml reaction solution (23) to 1 x 10^7 resident peritoneal cells plated/ml reaction volume. Thus, the macrophage concentration was 40 times that described by Ruch et. al. (23) To further modify Ruch's assay, the reaction volume was reduced from 2 ml to 0.2 ml to increase the concentration of H_2O_2.

Figure 5. Viability of T. cruzi after 2 hours in solutions containing phenol red or homovanillic acid, substrates for the assays measuring H_2O_2. Viability was determined by counting the remaining parasites.



*assay solution consists of: 140mM NaCl, 0.5mM $CaCl_2$, 5.5mM glucose, 10mM potassium phosphate buffer (pH 7.4)

Again, as with the modified phenol red assay, the optimal atmospheric conditions for the HVA assay was under either ambient air or 7% CO_2-ambient air (Figure 6). Since calcium and magnesium were included in Ruch's assay, the requirement for these metals in stimulation of optimal H_2O_2 production by PMA or opsonized zymosan was determined. When opsonized zymosan was the stimulant, addition of calcium or magnesium doubled the level of H_2O_2 produced. However, simultaneous additions of calcium and magnesium resulted in a 3.75 fold increase compared to the amount of H_2O_2 in the absence of calcium and magnesium. Thus, both calcium and magnesium were required for optimal H_2O_2 production (Figure 7). However, when PMA was the stimulant, either calcium or magnesium but not both were required for optimal H_2O_2 production (Figure 8). Addition of calcium or magnesium increased H_2O_2 production about 50%. Simultaneous addition of calcium and magnesium did not increase H_2O_2 production. This is in contrast to the results obtained with the phenol red assay where calcium was not required for optimal PMA stimulated H_2O_2 production. In addition the ability of NaN_3 to increase H_2O_2 measurements by inhibiting macrophage cytoplasmic catalase was again determined. Figure 9 shows that upon addition of 2 mM NaN_3, a concentration normally used to inhibit cellular catalase (25-32), H_2O_2 production was not increased suggesting that as before cytoplasmic catalase was not scavenging detectable amounts of H_2O_2.

The standard curve for the HVA assay was linear from 0 to 10 μM H_2O_2 (Figure 10). In this assay, H_2O_2 produced by resident macrophages was between the concentrations of 3 and 10 μM H_2O_2, or about

Figure 6. Modified HVA assay: Atmospheric conditions for optimal H_2O_2 production by adherent macrophages from 2 x 10^6 resident peritoneal cells. Macrophages were incubated with 6 or 12 μg opsonized zymosan or 1 or 10 μg PMA for 2 hours in 0.09 mM $CaCl_2$ and 0.05 mM $MgCl_2$.

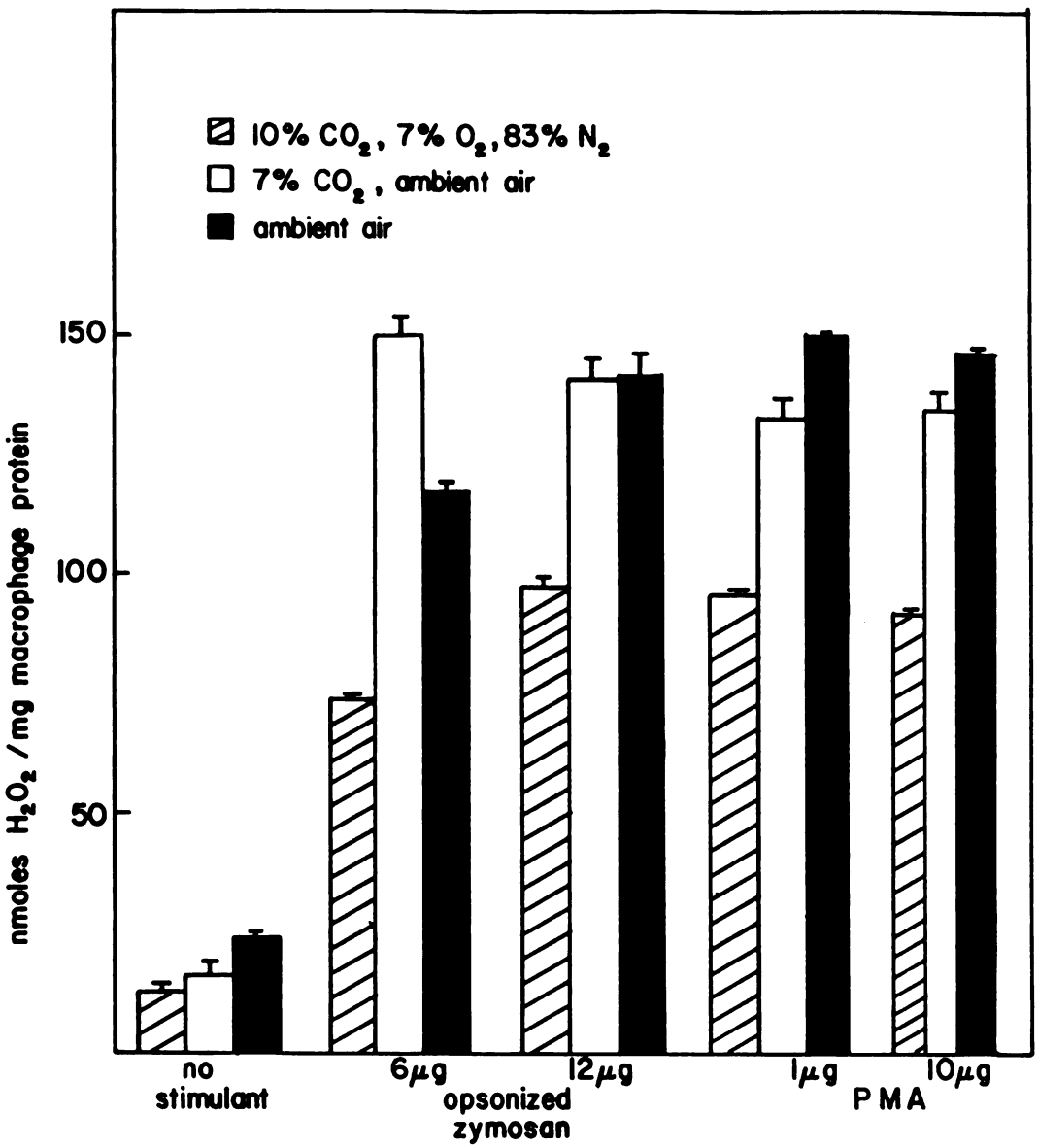

10% CO2, 7% O2, 83% N2
7% CO2, ambient air
ambient air
nmoles H2O2 /mg macrophage protein
150
100
50
no stimulant
6μg
12μg
opsonized zymosan
1μg
10μg
PMA

Figure 7. Modified HVA assay: Requirements for 0.09 mM $CaCl_2$ and 0.05 mM $MgCl_2$ for optimal H_2O_2 production by adherent macrophages from 2 x 10^6 resident peritoneal cells. Macrophages were incubated with 6 or 12 μg opsonized zymosan for 2 hours under 7% CO_2-ambient air.

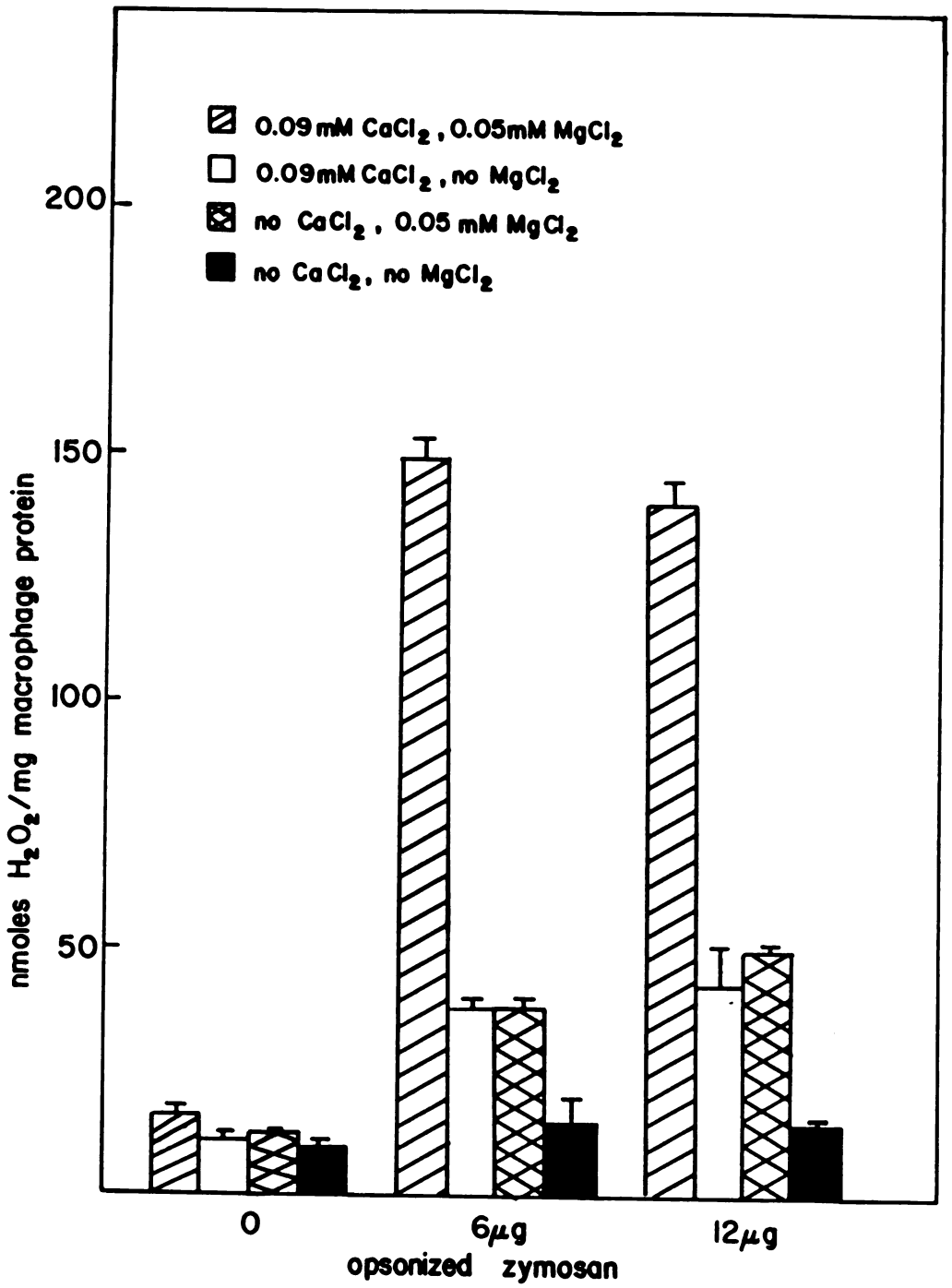

0.09 mM CaCl2 , 0.05mM MgCl2
0.09mM CaCl2 , no MgCl2
no CaCl2 , 0.05 mM MgCl2
no CaCl2 , no MgCl2
200
150
100
50
nmoles H2O2/mg macrophage protein
0
6μg
12μg
opsonized zymosan

Figure 8. Modified HVA assay: Requirements for 0.09 mM $CaCl_2$ and 0.05 mM $MgCl_2$ for optimal H_2O_2 production by adherent macrophages from 2 x 10^6 resident peritoneal cells. Macrophages were incubated with 1 or 10 μg PMA for 2 hours under 7% CO_2-ambient air.

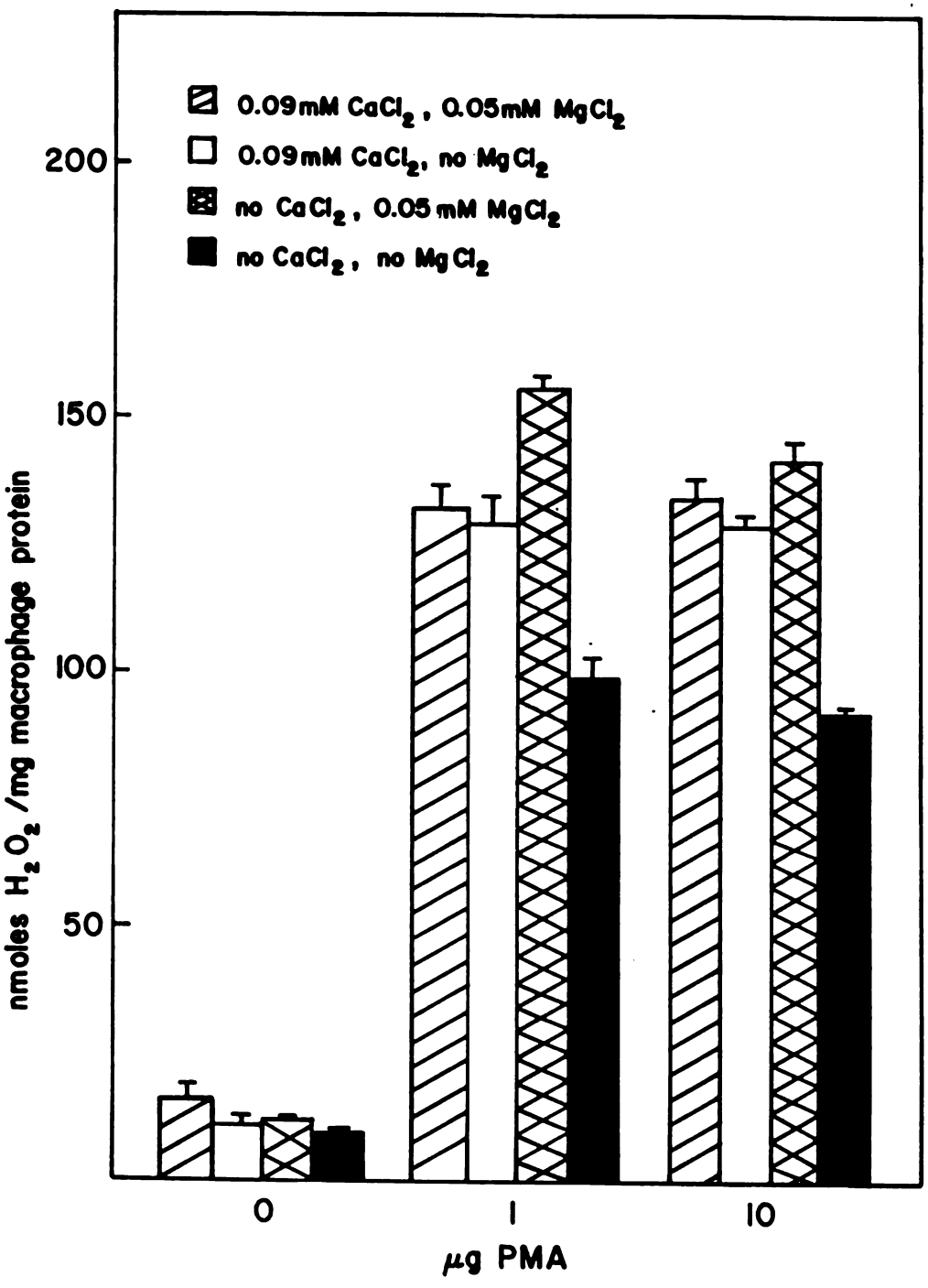

0.09mM CaCl2 , 0.05mM MgCl2
0.09mM CaCl2, no MgCl2
no CaCl2 , 0.05 mM MgCl2
no CaCl2 , no MgCl2
200
150
100
50
nmoles H2O2 /mg macrophage protein
0
1
10
µg PMA

Figure 9. Modified HVA assay: Requirements for 2 mM NaN_3 for optimal H_2O_2 production by adherent macrophages from 2 x 10^6 resident peritoneal cells. Macrophages were incubated with 6 or 12 μg opsonized zymosan or 1 μg PMA for 2 hours under 7% CO_2-ambient air.

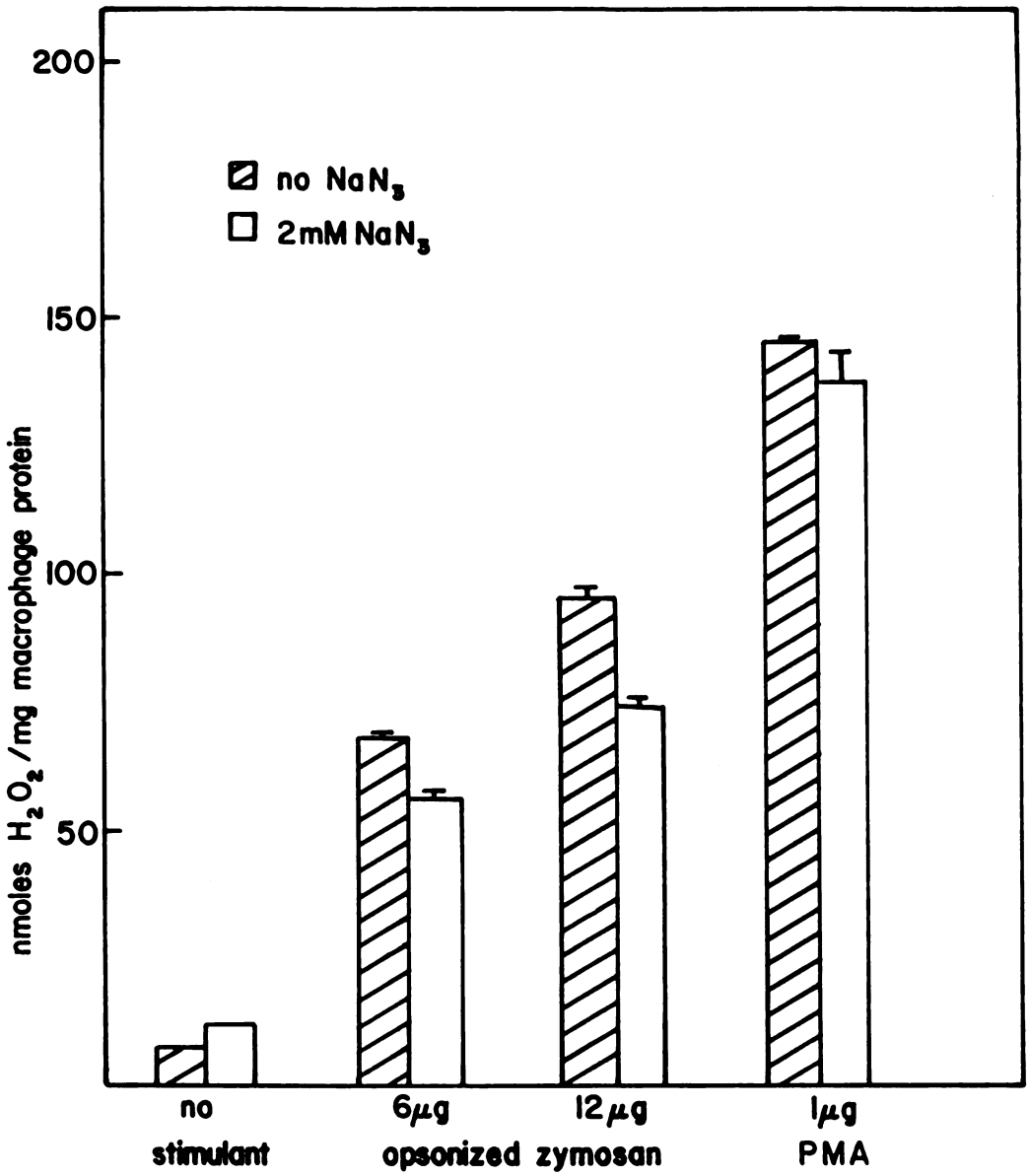
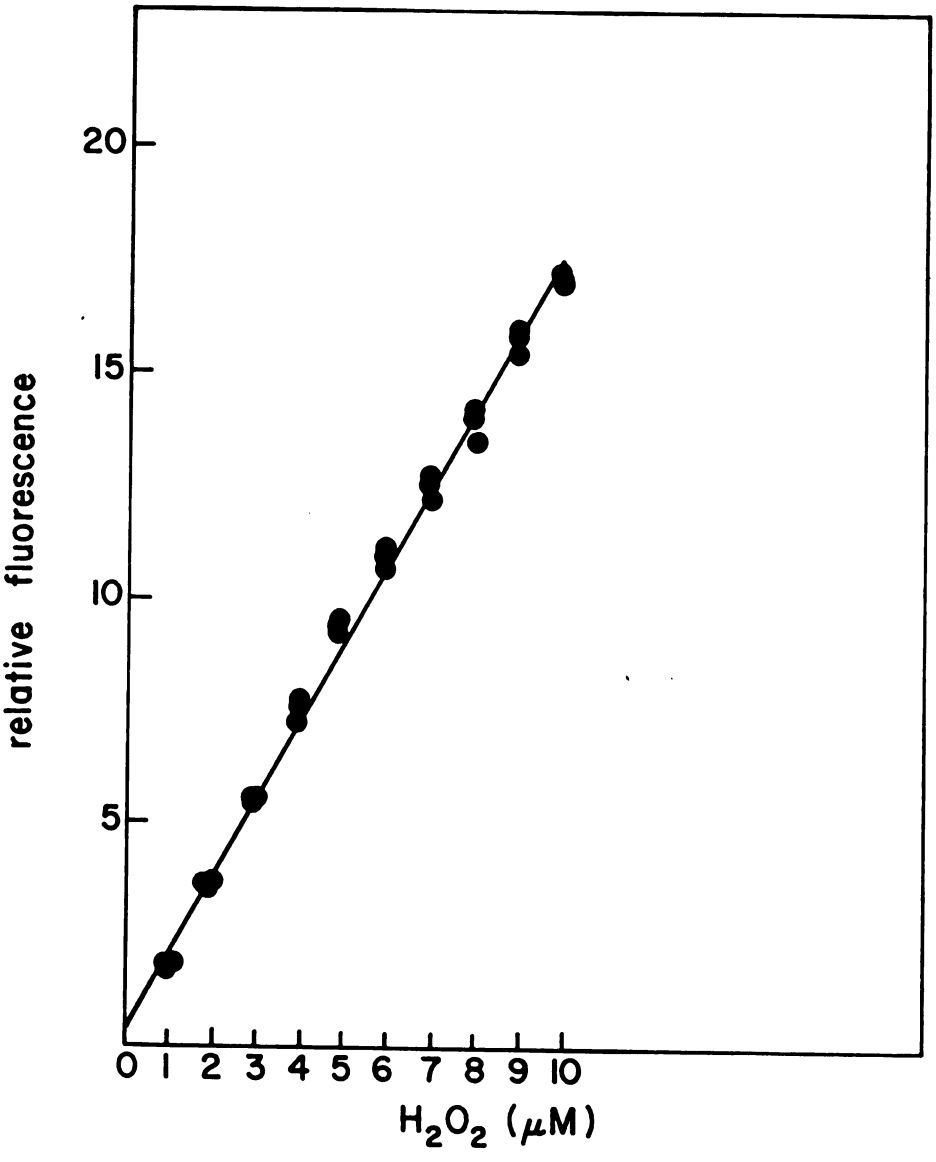

200
150
100
50
nmoles H_2O_2/mg macrophage protein
no NaN_3
2mM NaN_3
no stimulant
6μg opsonized zymosan
12μg opsonized zymosan
1μg PMA

Figure 10. Standared curve for H_2O_2 production in the modified HVA assay. Various concentrations of reagent H_2O_2 was incubated with the reaction solution for one hour since the reaction goes to completion by one hour and the product is stable for several hours.


relative fluorescence
20
15
10
5
0 1 2 3 4 5 6 7 8 9 10
H_2O_2 (μM)

40 to 150 nmoles H_2O_2 / mg macrophage protein (Figures 7 and 8). These H_2O_2 concentrations are within the linear portion of the standard curve. The total and extracellular concentrations of H_2O_2 (Figure 11) were also assessed for the modified HVA assay. As for the phenol red assay, the majority of H_2O_2 produced by either zymosan or PMA was found in the extracellular environment (Figure 11). As can be clearly seen in Figure 12, the modified HVA assay made it possible to demonstrate for the first time that T. cruzi could indeed initiate H_2O_2 production. In the first experiment where the parasites exhibited a high degree of infectivity, macrophages stimulated with nonopsonized T. cruzi generated about 30 nmoles of H_2O_2 per mg of macrophage protein. Resident macrophage infected with opsonized T. cruzi produced twice as much H_2O_2. Variation in infectivity of the parasites from experiment to experiment is common being a troublesome variable not yet under the control of parasitologists. At lower but nevertheless ample levels of infection (30-40%), a second experiment yielded a similar pattern of results. At a 10:1 ratio of T. cruzi to macrophages, the nonopsonized parasites caused production of half the amount of H_2O_2 as opsonized T. cruzi. Further, at the 10:1 ratio, there was definite correlations between the degree of infectivity of the T. cruzi and amount of H_2O_2 produced. In this, the parasites of experiment one which were 2.5 to 3.0 times more virulent, caused about 2.5 times more peroxide to be produced.

Figure 11. Modified HVA assay: Measurement of total, extracellular, and intracellular H_2O_2 produced by adherent macrophages from 2 x 10^6 resident peritoneal cells. Macrophages were incubated with 6 or 12 μg opsonized zymosan or 1 or 10 μg PMA in 0.09 mM $CaCl_2$, and 0.05 mM $MgCl_2$ under 7% CO_2-ambient air. Total H_2O_2 was measured by adding glycine-EDTA to stop the reactions to the cells and reaction solution. Extracellular H_2O_2 was measured by adding glycine-EDTA to the reaction solution after removal from the cells. Intracellular H_2O_2 was measured by adding fresh reaction solution to the remaining adherent macrophages and adding glycine-EDTA.

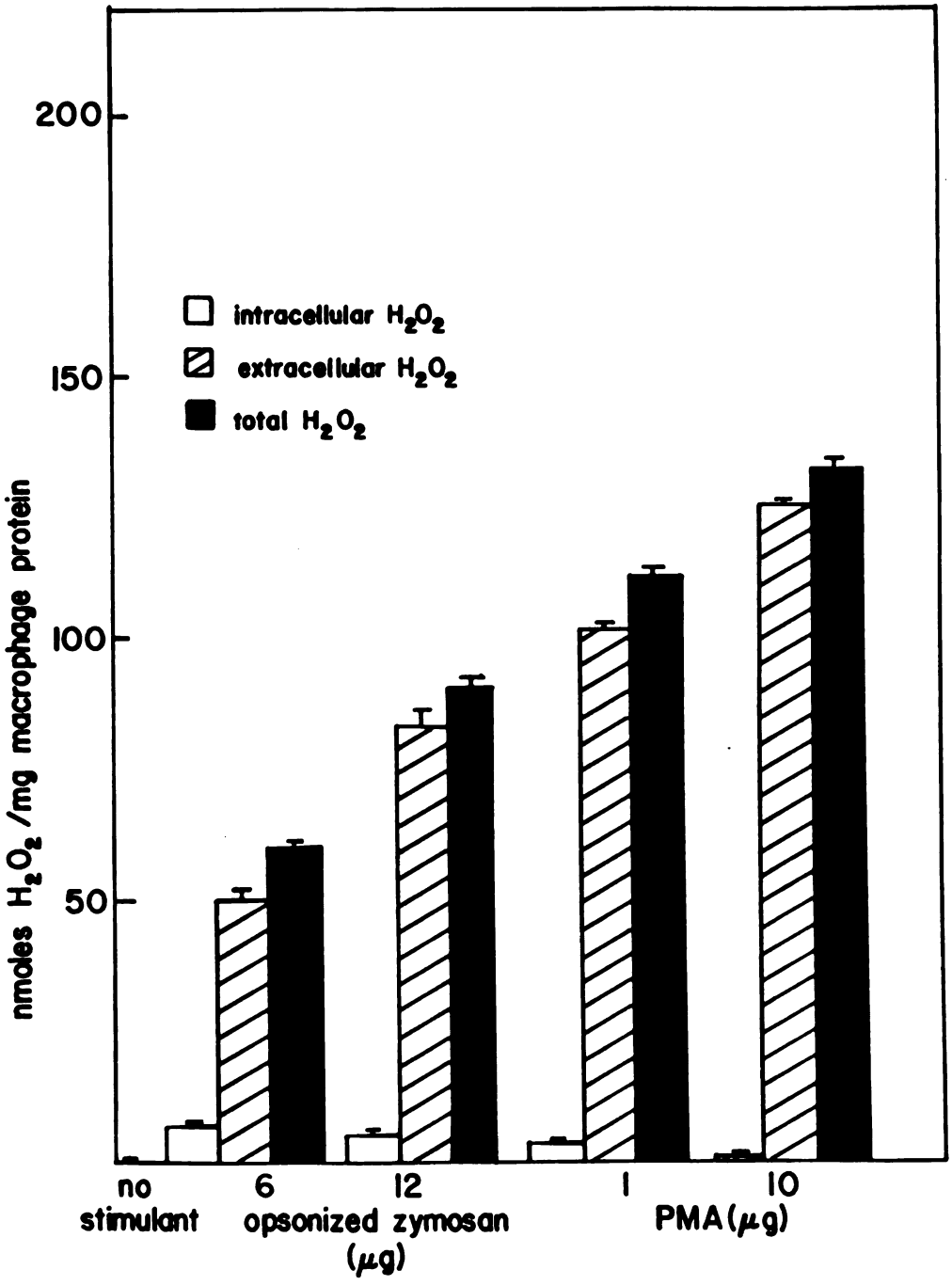
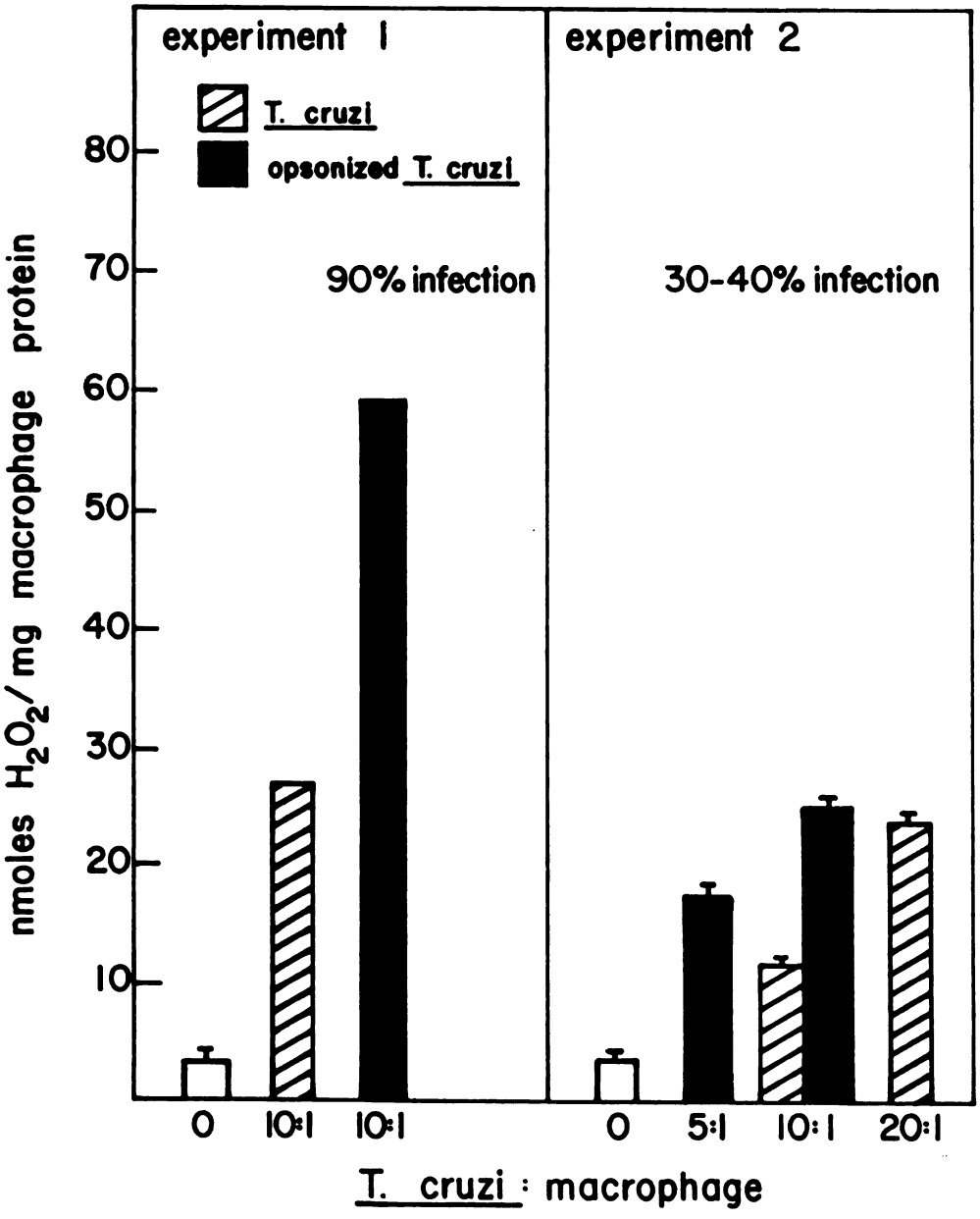

200
150
100
50
nmoles H_2O_2 /mg macrophage protein
intracellular H_2O_2
extracellular H_2O_2
total H_2O_2
no stimulant
6
12
opsonized zymosan (μg)
1
10
PMA(μg)

Figure 12. Modified HVA assay: H_2O_2 production by resident macrophages incubated with T. cruzi or opsonized T. cruzi at a parasite:macrophage ratio of 5:1, 10:1, or 20:1. H_2O_2 was measured after two hours incubation of parasites with adherent macrophages in the reaction solution with 0.09 mM $CaCl_2$ and 0.05 mM $MgCl_2$ under 7% CO_2-ambient air. T. cruzi and macrophages were incubated in DMEM for 2 hours and then the proportion of macrophages associated with parasites which is indicated as percent infection was determined. Protein content was determined for macrophages not incubated with parasites.


experiment 1
experiment 2
T. cruzi
opsonized T. cruzi
90% infection
30-40% infection
nmoles H_2O_2/mg macrophage protein
80
70
60
50
40
30
20
10
0
10:1
10:1
0
5:1
10:1
20:1
T. cruzi : macrophage

DISCUSSION

Modifications of the assay conditions for measurement of H_2O_2 production as described in this chapter have now made it possible to measure H_2O_2 production by resident macrophages. The concentration of resident peritoneal macrophages was increased in order to increase the amount of H_2O_2 produced in a given volume of assay solution. Thus, the concentration of the H_2O_2 and therefore the concentration of the phenol red product was increased. Also, the addition of 0.5 mM $CaCl_2$ increased the amount of H_2O_2 production when opsonized zymosan was the stimulant. This was probably due to the calcium requirement for cell phagocytosis since opsonized zymosan binds to receptors and is then phagocytosed. Most likely, other stimulants which involve receptor mediated phagocytosis will also require calcium for optimal H_2O_2 production. In contrast, calcium did not improve PMA stimulated H_2O_2 production. This is probably because PMA diffuses through the cell membrane thus not requiring calcium for receptor mediated phagocytosis. Also, in an attempt to increase H_2O_2 measurements, NaN_3 was added to inhibit macrophage cytoplasmic catalase which scavenges H_2O_2. However, addition of NaN_3 had no effect on the amount of H_2O_2 produced. This was predictable since murine macrophages contain relatively low to negligible levels of catalase activity as compared to macrophages from humans or guinea pigs (31). When studing macrophages from other species, it is important to add NaN_3 to inhibit scavenging of H_2O_2 by cytoplasmic catalase. Also,

optimal H_2O_2 production was obtained under ambient air. This is probably a result of the presence of a greater supply of oxygen which is the substrate for NADPH oxidase. In some previous studies where the oxygen supply was low due to use of other atmospheric conditions (5-9), there was probably an underestimate in the amount of H_2O_2 that the cells were capable of producing. Thus, many previous reports suggest that very small to negligable amounts of H_2O_2 are produced by resident macrophages (2, 5, 6, 7, 8, 9). In our hands, similar results were obtained using the published assay conditions (0-7 nmoles H_2O_2 / mg macrophage protein). In contrast, using the modified phenol red assay described in this chapter, much higher amounts of H_2O_2 were produced by resident macrophages (40-60 nmoles H_2O_2 / mg macrophage protein). So, with an increase in cell concentration, addition of calcium, and incubation under ambient air, the amount of H_2O_2 produced by resident macrophages is increased to the middle of the standard curve. Thus, these modifications now make it possible to measure H_2O_2 production by resident macrophages.

Additional modifications of the assay for H_2O_2 production were made for use with the parasitic stimulant, T. cruzi. Since the substrate, phenol red, was toxic to the parasite, another nontoxic substrate, HVA, was used. Ruch et. al. (23) described the rarely used HVA assay. The conditions for this assay were also improved by increasing the cell concentration when using resident macrophages. In addition, since Ruch's assay included calcium and magnesium in the reaction solution, the requirements for these in optimal stimulation of H_2O_2 production were determined. Calcium and magnesium were

required for optimal opsonized zymosan stimulated H_2O_2 production. However, only calcium or magnesium but not both were required for optimal PMA stimulation. The calcium requirement for opsonized zymosan stimulation was probably due to the need for calcium for phagocytosis of the opsonized zymosan. This modified HVA acid assay was more sensitive than the phenol red assay since lower concentrations of H_2O_2 could be measured.

Using the modified HVA assay for H_2O_2, T. cruzi trypomastigotes produced from 10-30 μmoles H_2O_2 depending on ratio of T. cruzi to mononuclear cells and degree of infectivity of the parasite. Those which had been opsonized with chronic serum stimulated twice as much H_2O_2 production as nonopsonized trypomastigotes. This would make sense since once the immune system mounts an antibody response to the parasite, trypomastigotes (the blood form of T. cruzi) may actually be opsonized in vivo. Generally speaking, the second phase of any immune response is more intense. The HVA assay can now be used to answer questions about the role of opsonization in parasitic defense. For the most part, the amount of T. cruzi stimulated H_2O_2 production depended upon the amount of T. cruzi present and degree of infection. This is the first instance where T. cruzi stimulated H_2O_2 production has been directly and quantitatively measured, since as pointed out in the introduction, previous assessments had been done indirectly or nonquantitatively (12-14).

The HVA assay will be a vital addition to immunoparasitology by making it possible to answer many key questions regarding the killing of T. cruzi by mononuclear cells. The question of whether or not

H_2O_2 is a key element in the killing of T. cruzi can now begin to be addressed. It will also be important to know what the relationship is between the amount of H_2O_2 produced and the number of parasites that can associate with a particular phagocytic cell. In other words, as the burden of potentially infective T. cruzi increases, can the macrophage respond with incremented increases in H_2O_2 production. If this is so, is the amount of H_2O_2 produced directly proportional to the degree of associated parasites or are there limitations and thresholds that might explain why Chagas' disease is never completely eliminated by the immune system. Assessment of the ability of the various life-cycle forms of T. cruzi to initiate H_2O_2 production might shed additional light on the ability of the various forms to escape the host defense system. Finally, the ability of the various forms of the mononuclear cells to produce H_2O_2 upon stimulation by T. cruzi can also be evaluated. Thus comparisons in intensity of H_2O_2 production between resident and peritoneal elicited macrophages and peripheral or tissue-localized phagocytes can be made.

Finally, this assay made it possible to do the studies in chapter 5 of this thesis. There, it was essential that T. cruzi stimulate measurable quantities of H_2O_2 production by resident peritoneal macrophages so that the deficit in killing T. cruzi by zinc deficient resident macrophages could be investigated. Also, if H_2O_2 production was reduced in T. cruzi stimulated zinc deficient macrophages, it may help identify the zinc dependent step(s) in the mechanism(s) of parasite destruction.

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Chapter 4

T. cruzi-STIMULATED H_2O_2 PRODUCTION: QUANTITATION AND A POSSIBLE MECHANISM

ABSTRACT

For the first time, Trypanosoma cruzi (T. cruzi)-stimulated H_2O_2 production by leukocytes was directly quantitated. Assessment of the ability of leukocytes to produce H_2O_2 is very important since H_2O_2 is thought to be crucial in the destruction of T. cruzi parasites. Blood forms of T. cruzi (trypomastigotes) that were opsonized with heat inactivated serum from chronically infected mice stimulated four times as much H_2O_2 production by resident macrophages as nonopsonized trypomastigotes. If the intracellular form of T. cruzi, the amastigote, was the stimulant, half as much H_2O_2 was produced by resident macrophages as compared to stimulation by the trypomastigote. Further, it was demonstrated that H_2O_2 production stimulated by trypomastigotes and opsonized trypomastigotes but not amastigotes correlated with the level of parasite-macrophage association. Moreover, an increase in H_2O_2 production coincided with an increase in killing of trypomastigotes, amastigotes, or opsonized trypomastigotes. The amount of H_2O_2 produced when 50% of the trypomastigotes or amastigotes were destroyed was the same. When 50% of the opsonized trypomastigotes were killed about 3.5 times as much H_2O_2 was produced.

H_2O_2 production was characterized further by investigating a possible mechanism or route for intiation of T. cruzi stimulated H_2O_2 production. The following preliminary results suggest that arachidonate metabolites are second messengers in the activation of H_2O_2 production by T. cruzi. The macrophage phospholipids contained a considerable amount of arachidonate as a source for production of

these arachidonate metabolites; 21% of the fatty acids were arachidonate. Also, addition of T. cruzi to resident macrophages preincubated with ^{3}H-arachidonate stimulated the release of PGE_2, HETE's and what may be leukotrienes at a ratio of 2:1:4. It is known that HETE's and leukotrienes but not PGE_2 stimulate resident macrophages to produce H_2O_2. If T. cruzi also stimulates release of other fatty acids that have shorter chain length or less saturation than arachidonate (20:4), they would not activate nearly as much H_2O_2 production as 20:4. Furthermore, exogenous 20:4 was shown to stimulate 2 to 3 times as much H_2O_2 production as the exogenous addition 18:3, 18:2, or 18:0. In summary, since T. cruzi stimulates release of HETE's, what may be leukotrienes, and H_2O_2 and since HETE's and leukotrienes activate H_2O_2 production, HETE's and leukotrienes may be second messengers for T. cruzi stimulated H_2O_2 production by resident macrophages.

INTRODUCTION

Previous studies have suggested that Trypanosoma cruzi (T. cruzi) stimulates phagocytic cells of the immune system to produce H_2O_2 and that H_2O_2 is critical for the destruction of T. cruzi. H_2O_2 production by infected cells has been demonstrated qualitatively by a cytochemical stain for H_2O_2 (1); the stain was present in macrophage vacuoles containing T. cruzi. However, it had never been demonstrated in a direct, quantitative way that T. cruzi could indeed initiate H_2O_2 production. Instead, the chemical phorbol myristate acetate (PMA) was used to stimulate H_2O_2 production by activated macrophages and then the H_2O_2 production was mathematically correlated with destruction of T. cruzi by activated macrophages as determined in another assay (2). In a further attempt to demonstrate that H_2O_2 was important to the destruction of T. cruzi, killing of T. cruzi by inflammatory cells has been inhibited by the H_2O_2 scavenger, catalase, but not by inhibitors of other oxygen metabolites (2, 3). Chapter 3 demonstrated that T. cruzi does in fact stimulate quantitifiable amounts of H_2O_2 (10-30 nmoles H_2O_2/ mg macrophage protein). Whether or not the amount of H_2O_2 produced correlated with the number of T. cruzi associated with the macrophages remained unknown. Therefore, to further characterize H_2O_2 production by T. cruzi infected macrophages, studies were done herein to assess the amount of H_2O_2 produced by resident macrophages as related to the levels of infection of macrophages by several forms of the Tulahuén strain of T. cruzi. Also, correlations were done herein

between T. cruzi-stimulated H_2O_2 production and destruction of T. cruzi in an attempt to determine whether or not H_2O_2 was indeed critical to the killing of T. cruzi by macrophages.

The mechanism by which T. cruzi might initiate H_2O_2 production by macrophages is also unknown. Mechanisms of stimulation by agents other than T. cruzi such as PMA and opsonized zymosan have been identified. PMA diffuses into the cell membrane and activates protein kinase C to phosphorylate NADPH oxidase which in turn catalyzes production of O_2^-; O_2^- dismutates to H_2O_2 (4-11). On the other hand, opsonized zymosan enters the macrophage through receptor(Fc)-mediated phagocytosis and stimulates the release of arachidonate which then activates NADPH oxidase (6, 7, 12, 13, 14, 15, 16). The mechanism(s) for T. cruzi stimulated H_2O_2 production has not been identified. Identification of the mechanism(s) whereby T. cruzi activates H_2O_2 production was difficult since the mechanism for T. cruzi invasion of macrophages and the receptors on macrophages for phagocytosis of T. cruzi are unknown. In this chapter, information that begins to elucidate a possible mechanism for T. cruzi stimulated H_2O_2 production by macrophages is presented. Since exogeneous addition of LTC_4 or LTB_4 has been shown to increase killing of T. cruzi by macrophages (17, 18) and since T. cruzi is phagocytosed as is opsonized zymosan, it seems probable that 20:4 or 20:4 metabolites might also be second messengers in T. cruzi-stimulated H_2O_2 production. Therefore, release of 20:4 and its metabolites by ^{3}H-20:4 labelled resident macrophages incubated with T. cruzi was measured. The endogenous 20:4 content of the phospholipids of resident

macrophages from A/J mice was analyzed. Also, since other fatty acids may be released from macrophage phospholipids upon infection with T. cruzi, the ability of several exogenously added fatty acids to activate H_2O_2 production by resident macrophages from A/J mice was compared.

MATERIALS AND METHODS

Animals. A/J female mice were purchased from Jackson Laboratory, Bar Harbor Maine. Female Crl-CD-1 (IRC)BR Swiss mice were purchased from Charles Rivers, Portage, Michigan.

Isolation of Trypanosoma cruzi (T. cruzi). Four week old Crl-CD-1 (IRC)BR Swiss female mice were infected intraperitoneally with 2×10^5 blood forms (trypomastigotes) of the Tulahuèn strain of T. cruzi. Twelve to 14 days later, blood was collected from the axillary artery of mice anesthetized with ether. Blood was collected in tubes containing heparin or disodium ethylene-diaminotetracetate (EDTA) powder so that the final concentrations were 25 U/ml or 2.5 mg/ml, respectively. Parasites from the blood were separated on a lympholite gradient (isolymph, Gallard-Schlesinger, Carle, N.Y.) (19) and passed through a diethylaminoethylcellulose column (20). The parasites were centrifuged (800 x G, 15 minutes, 4°C) and resuspended in Dulbecco's modified minimal essential medium (DMEM, Gibco, Grand Island, NY) supplemented with 100 IU penicillin and 100 µg streptomycin / ml. One hundred percent of the cells were viable parasites in the trypomastigote form as determined by morphology and motility. Before use in the assays for H_2O_2, the parasites were washed two times in phosphate buffered saline (PBS) - 1% glucose and resuspended in the reaction solution for the H_2O_2 assay.

Amastigotes were collected from the supernatants of T. cruzi-infected rat heart myoblast cultures as previously described (21).

Preparation of opsonized T. cruzi. T. cruzi trypomastigotes

were opsonized by incubation for 1 hour at 37°C in 10 mM PBS (pH 7.4) with 1% glucose and 20% complement inactivated serum from chronic T. cruzi infected CD1 mice. The opsonized T. cruzi were washed 2 times and resuspended in 10 mM PBS (pH 7.4). The serum was obtained during the chronic phase of experimental Chagas' disease which occurs 5-6 weeks after the initial infection. Chronic mice have normal immune responsiveness but continue to harbor the parasite and produce T. cruzi-specific antibodies (22, 23).

Collection, isolation, and identification of peritoneal macrophages. Cells were harvested from the unelicited mouse peritoneum by lavage with 5 ml of cold (4°C) DMEM and 0.5% w/v gamma globulin free bovine serum albumin (BSA, Calbiochem, La Jolla, CA) (24). Cells were adhered to tissue culture plates for 1 1/2 hours at 37°C and 7% CO_2-ambient air. Nonadherent cells were removed by washing with 37°C DMEM. The percentage of macrophages/monocytes adhered to the culture plate was determined by staining for nonspecific esterase activity as detailed in the modification (25) of the technique described by Yam et. al. (26). Always, 94-100% of the adherent cells were identified as monocytes/macrophages by staining for nonspecific esterase and by morphology.

Homovanillic acid assay for H_2O_2. The assay conditions described by Ruch et. al. (27) were modified for use with resident macrophages and the stimulant, T. cruzi trypomastigotes. One milliliter of 2 x 10^6 resident peritoneal cells/ ml was added to a 24 well flat bottom plate (Corning) and allowed to adhere for 2 hours. Nonadherent cells were removed from the adherent macrophage monolayer by washing

three times with PBS - 1% glucose (37°C). Two hundred microliters of reaction solution which consisted of PBS, 1% glucose, 0.09 mM $CaCl_2$, 0.05 mM $MgCl_2$, 200 μM homovanillic acid, and 2 U/ml horseradish peroxidase were added per well. The fatty acid stimulants 20:4, 18:3, 18:2, or 18:0 were diluted in ethanol and 2 μl added per well so that less than 1% of the reaction solution was ethanol and the final concentration was 10 μM or 50 μM. When parasites were the stimulant, they were resuspended in the 200 μl of reaction solution to be added to the macrophages. Standards consisted of wells containing reaction solution and 0-20 μM H_2O_2 but no cells. Nonspecific production of H_2O_2 was determined by including wells containing cells but no stimulant or stimulant but no cells. The plates were centrifuged at 50 x G for 3-4 minutes to increase parasite-macrophage interactions and incubated for 90 minutes in a humidified atmosphere at 37°C and 7% CO_2-ambient air. The reaction was stopped by the addition of 25 μl of 25 mM EDTA and 0.1 M glycine at pH 12. The relative fluorescence of the homovanillic acid dimer was determined using a spectrofluorometer (Perken-Elmer 650-40) equipped with a microcuvette. The excitation and emission wavelengths were 312 nm and 420 nm, respectively. The slit widths for excitation and emission were 2 nm and 5 nm, respectively. The amount of H_2O_2 in the samples was analyzed by linear regression of the standard curve. The amount of macrophage protein was determined by the method described by Lowry (28). The data are presented as nmoles H_2O_2 / mg macrophage protein. Nmoles of H_2O_2 / mg macrophage protein was calculated as follows: (μM H_2O_2 calculated from the standard curve) x (1

liter/ 1000 ml) x (0.225 ml final volume of reaction solution) x (1000 nmoles/ 1μmole)/(mg macrophage protein determined from the Lowry) = nmoles H_2O_2 / mg macrophage protein.

Association of T. cruzi with macrophages. After incubating the macrophages with parasites for 90 minutes in DMEM at 37°C and 7% CO_2-ambient air, the nonassociated parasites were removed by washing three times with PBS - 1% glucose. The cultures were incubated in DMEM for another 18 hours and/or fixed with methanol for 5 minutes, allowed to dry, and stained for 1 hour with Giemsa in 10 mM phosphate buffer (pH 6.8). The cells were washed one time with 10 mM phosphate buffer (pH 6.8). Numbers of parasites per 100 macrophages and percent of macrophages associated with parasites were determined by counting at least 200 cells per replicate. The percent decrease in number of T. cruzi associated with macrophages from the time of removal of nonassociated parasites to 19 to 24 hours later represented killing of T. cruzi by macrophages. At 19 to 24 hours, the number of nonassociated parasites was less than 3% of the number of parasites associated with the macrophages. Thus, a reduction in number of parasites associated with the macrophages was due to killing and not simply escape of the parasites from the macrophages.

Synthesis of margaric phosphatidylcholine (29). Margaric (17:0) phosphatidylcholine was chosen as an internal standard for the analysis of fatty acid composition of macrophage phospholipids since methylated margaric acid has a different retention time during gas liquid chromatography than fatty acids normally found in good quantities in mammalian phospholipids. Margaric acid (200 mg) was placed

in a small teflon capped tube with 300 µl thionyl chloride, pulsed with nitrogen, and placed on a steam bath for 1 hour. The products were evaporated to dryness under nitogen in a 80°C water bath, washed successively with water, 10% sodium carbonate, and water, and then dried under nitrogen. The fatty acyl chloride (50 mg) was reacted with the $CdCl_2$ complex of L-alpha-glycerophosphorylcholine (10.45 mg) while stirring for 2 hours in 150 µl chlorform and 9 µl pyridine under an atmosphere of nitrogen. The stirrer was rinsed with chloroform and the solvents were evaporated under nitrogen. The reaction products were dissolved in 166 µl ether (dry), cleared by centrifugation, and the solvents removed under nitrogen. The lipids were dissolved in 500 ul acetone (dry), placed on a acetone-CO_2 bath for 1 hour and isolated by centrifugation in the cold. They were precipitated again with 250 µl acetone. The lipids were dried under nitrogen, dissolved in chloroform:methanol:water (5:4:1, v:v:v) and passed over a 2ml column of amberlites IR50:IRC50 (50: 50). The column was washed with the solvent mixture. The effluent was dried under nitrogen, dissolved in chloroform and placed over a 1 ml silicic acid column. The silicic acid column was washed with 30 column volumes of chloroform and the phospholipid was eluted with 15 column volumes of chloroform:methanol (1:1) followed by 10 column volumes of methanol. The eluent was evaporated under nitrogen and the phospholipid was stored dry under nitrogen at -20°C. The last 5ml fraction of the silicic acid column effluent did not contain margaric acid as measured by gas liquid chromatography of the fatty acid methyl ester. Esterification is described below. The phospholipid

fraction from the silicic acid column contained margaric acid as determined by gas liquid chromatography of the methyl ester.

Fatty acid composition of macrophage phospholipids. Macrophages were adhered for 2 hours to 24 well tissue culture plates in DMEM-0.5% BSA. Nonadherent macrophages were removed by washing three times in DMEM-0.5% BSA. The adherent macrophages were collected by scraping into 0.15M NaCl. Since one twelfth of the amount of the synthesized internal standard, margaric phosphatidlycholine, gave a peak height during gas liquid chromatography that was 80% of the chart range, this amount of internal standard was added to each sample to control for sample loss during the purification of the macrophage phospholipids. The lipids were extracted by the method described by Bligh and Dyer (30). Briefly, methanol:chloroform:water (4:2:8, v:v:v) was added to the macrophages. Then one volume of chloroform followed by one volume of water was added to the extraction mixture. The mixture was centrifuged and the chloroform layer was collected and evaporated under nitrogen. The lipids were resuspended in chloroform and placed over a 0.5 ml heat activated silicic acid column in a pasteur pipette. The neutral lipids which do not bind to the column were washed through the column with chloroform. The phospholipids bound to the column were then eluted with chloroform:-methanol (1:1) followed by methanol. The organic solvents were evaporated from the neutral and phospholipids under nitrogen. The fatty acids were converted to methyl esters (31) by incubating overnight at 75°C in 6% HCl in methanol under nitrogen. The fatty acid methyl esters were extracted with three equal volumes of hexane and

washed once with water. Water was removed from the hexane by the addition of anhydrous sodium sulfate. The hexane was collected and evaporated under nitrogen. The fatty acid methyl esters were dissolved in hexane and analyzed (32) using a Hewlett-Packard gas chromatograph equipped with a 6 foot x 1/8 inch i.d. glass column packed with 10% SP-2330 Chromasorb W/AW. Analysis was performed isothermally at 180°C for 7 minutes followed by an increase of 4°C /minute to a limit of 200°C. The carrier gas-flow rate was 30 ml/minute. Peak identifications were based on relative retention times of standard methyl esters (Nu Chek Prep, Elysian, Minnesota). Relative peak areas were measured by a Hewlett-Packard 3380A electronic integrator. The quantity of the various fatty acids was obtained using linear regression from a standard curve of peak area versus amount of fatty acid methyl ester. Total macrophage fatty acid was determined by comparing peak area of the internal standard in the samples of macrophage phospholipids to peak area of a sample of internal standard alone that had not been submitted to the phospholipid isolation procedure. Also, samples of macrophage phospholipids from each severely zinc deficient, moderately zinc deficient, restricted, and zinc adequate mice, which did not contain the internal standard, did not have a peak with the same retention time as the internal standard. Thus, since the macrophage phospholipids did not contain margaric acid, margaric acid was a valid internal standard.

Arachidonic acid release by macrophages (33, 34). Macrophages adhered to 24 well plates were incubated with 1.5 μCi (5, 6, 8, 9, -11, 12, 14, 15-^{3}H)arachidonic acid (Amersham) in 2 ml DMEM overnight

and washed three times with serum free DMEM at 37°C . The macrophages were stimulated by nonopsonized trypomastigotes of T. cruzi for 2 hours in serum free DMEM. Nonstimulated macrophages were included to control for spontaneous release of radioactivity by the macrophages. The culture medium was removed from the adherent macrophage monolayer, centrifuged, and acidified to 0.03M with citric acid. The acidified media was extracted three times with 3 ml of chloroform:methanol (2:1, v:v). The chloroform layers were combined, washed two times with 2 ml of methanol:water (2:1, v:v) and evaporated to dryness under nitrogen. The residue was dissolved in 40 µl of ethylacetate:methanol (3:1, v:v) and applied to precoated plates (Silica Gel 60, Merck) for thin-layer chromatography. The internal standards PGE_2 and arachidonate were added to each sample. Other standards 6-keto-PGF_{1alpha}, LTB_4, LTC_4, LTD_4, LTE_4, and ricinoleic acid (an oxidized product of arachidonate) were run separately along with PGE_2 and arachidonate. Arachidonate and its metabolites were separated by development twice in the solvent system chloroform:ethylacetate:methanol:acetic acid:water (140:60:16:2:1, v:v:v:v:v). The internal standards were visualized by exposure to I_2. Half centimeter segments for each ascending sample of the silicic acid coated plates were scraped and radioactivity was measured by a Delta 300 scintillation counter (Tracor Analytic). Radioactivity for each segment of a sample was plotted versus distance (cm) on the plate. The data for each radioactive peak are presented as ^{3}H-cpm of stimulated release minus ^{3}H-cpm spontaneous release from nonstimulated samples.

^{3}H-arachidonic acid labelled macrophage phospholipids. The methods for identification of those macrophage phospholipids that contained ^{3}H-arachidonate used herein was described by Emilsson et. al. (35, 36). Briefly, radiolabelled resident macrophages were collected by scraping into 1 ml of ice-cold 10 mM HCl. Lipids were extracted with 6 ml of chloroform:methanol (1:1, v:v). Phase separation was obtained by addition of 2 ml of 10 mM HCl followed by centrifugation. The chloroform layer was removed, evaporated to dryness, and dissolved in 40 µl ethylacetate:methanol (3:1, v:v). The lipids were separated by thin layer chromatography on precoated plates (Silica Gel 60, Merck) with the solvent system chloroform:methanol-:acetic acid:water (25:20:3:0.3, v:v:v:v). Lipids were visualized by exposure to I_2. The plates were scraped in 0.5 cm segments for each ascending sample and radioactivity was measured by a Delta 300 scintillation counter (Tracor Analytic). Radioactivity for each segment of a sample was plotted versus distance on the plate. The total radioactivity for each peak was calculated. The data for each radioactive peak are presented as radioactivity released by T. cruzi stimulated macrophages minus spontaneous release of radioactivity by nonstimulated macrophages.

Macrophage viability. Viability of the adherent macrophages was determined using the trypan blue exclusion method. Always >95% of the macrophages were viable.

RESULTS

For the first time, it was possible to demonstrate that measurable amounts of H_2O_2 could be detected in cultures of macrophages stimulated with T. cruzi trypomastigotes using the modified homovanillic acid assay for H_2O_2 (Figure 1). The increased sensitivity of this assay made it possible to use resident macrophages instead of the activated macrophages as was normally used in the past. Thus, T. cruzi trypomastigotes were capable of stimulating the generation of H_2O_2 by macrophages. This is important since H_2O_2 is thought to destroy T. cruzi. Previously, indirect evidence suggested that macrophages produced H_2O_2 in response to T. cruzi since it has been shown that T. cruzi stimulates the uptake of oxygen (37), that a cytochemical stain for H_2O_2 is present in vacuoles containing T. cruzi (1), and that catalase, the scavenger for H_2O_2 abrogates killing of T. cruzi by macrophages. Finally, herein, T. cruzi has been directly shown to stimulate H_2O_2 production in measurable quantities.

To determine if the amount of H_2O_2 produced correlated with macrophage infection, the percentage of macrophages associated with trypomastigotes and the number of parasites per 100 macrophages from several experiments were compared with the macrophage production of H_2O_2 (Figures 1 and 2). As the percentage of macrophages associated with the trypomastigotes increased, there was a linear increase in H_2O_2 production (correlation coefficient = 0.957, Figure 1). When trypomastigotes were opsonized with chronic serum, a similar correlation was found between parasite association with macrophages and

Figure 1. Correlation between H_2O_2 production and proportion of macrophages associated with T. cruzi. For measurement of H_2O_2 production, T. cruzi amastigotes, T. cruzi trypomastigotes, or opsonized T. cruzi trypomastigotes were incubated for 2 hours with adherent macrophages in in the reaction solution. Parasites were incubated for 2 hours with macrophages in DMEM and then the proportion of macrophages associated with T. cruzi was determined. The points for each form of parasite were from experiments performed on several days with freshly prepared macrophages, parasites, and reaction solution.

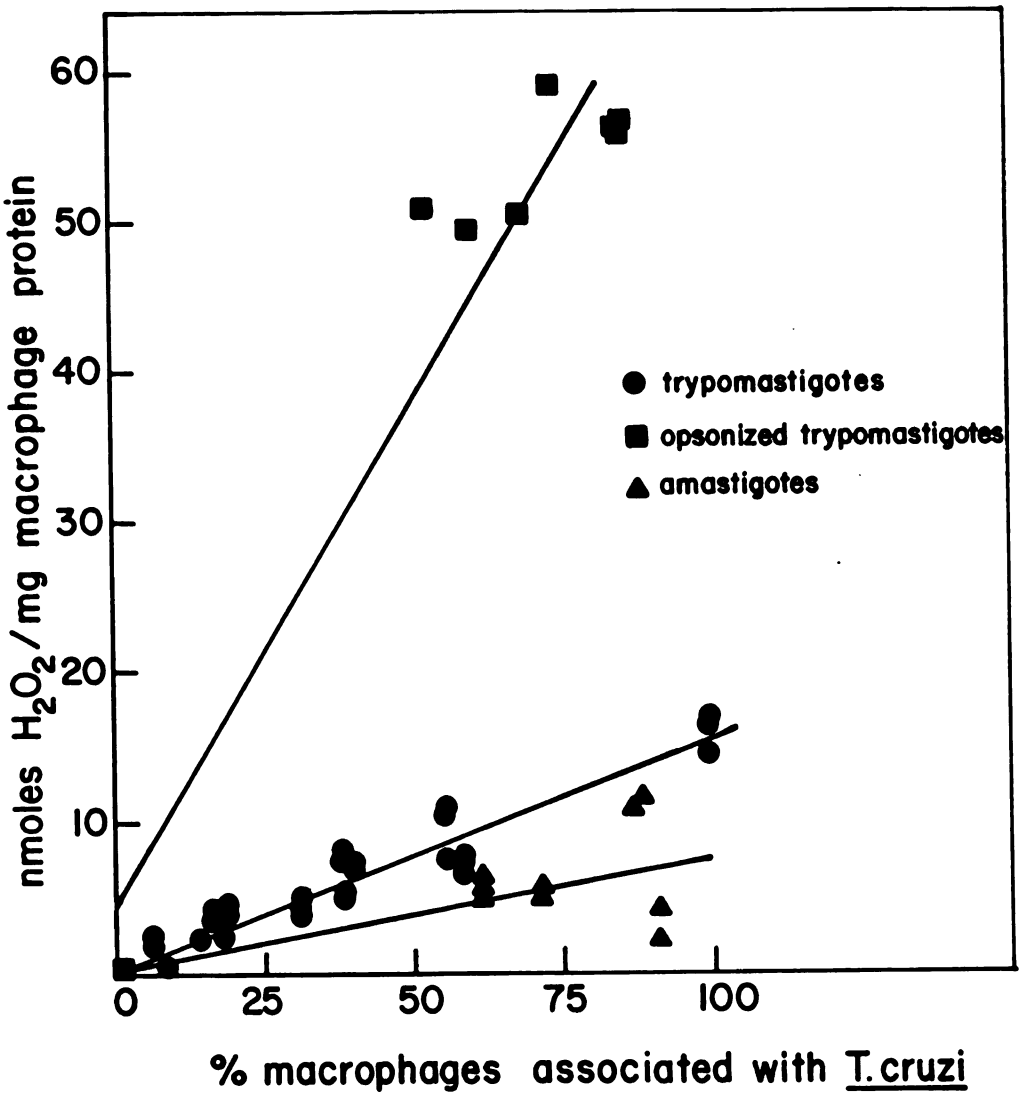
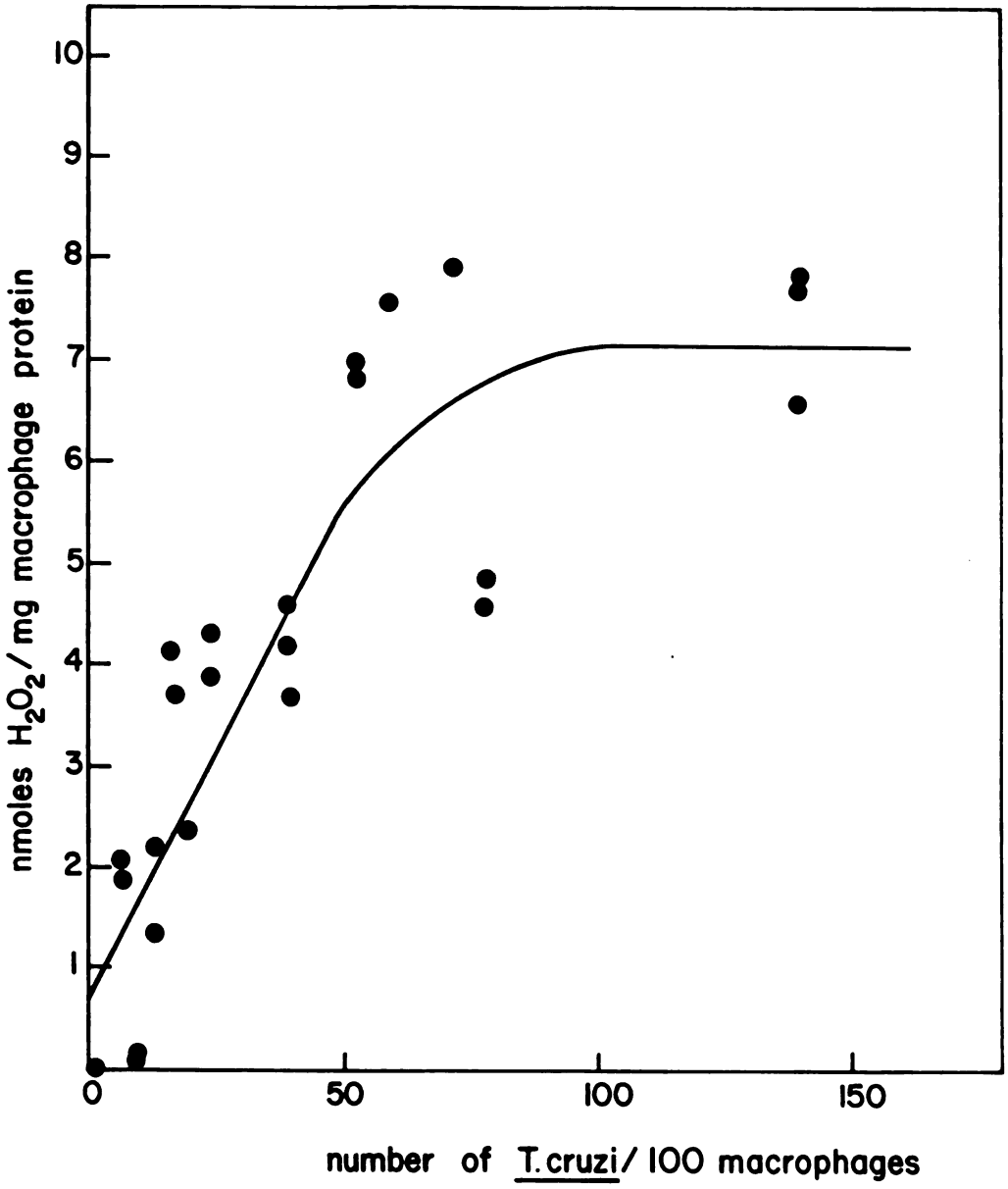

60
50
40
30
20
10
nmoles H_2O_2/mg macrophage protein
0
25
50
75
100
% macrophages associated with T.cruzi
trypomastigotes
opsonized trypomastigotes
amastigotes

Figure 2. Correlation between H_2O_2 production and number of T. cruzi per 100 macrophages at low levels of T. cruzi-macrophage association. For measurement of H_2O_2 production, T. cruzi trypomastigotes were incubated for 2 hours with adherent macrophages in the reaction solution. Parasites were incubated for 2 hours with macrophages in DMEM and then the number of T. cruzi associated with macrophages was determined. The points were from experiments performed on several days with freshly prepared macrophages, parasites, and reaction solution.


10
9
8
7
6
5
4
3
2
1
0
50
100
150
nmoles H_2O_2 / mg macrophage protein
number of T.cruzi / 100 macrophages

production of H_2O_2 (correlation coefficient = 0.950, Figure 1). However, opsonized trypomastigotes stimulated considerably more H_2O_2 production than did nonopsonized trypomastigotes (Figure 1). For example, when 50% of the macrophages were associated with parasites, opsonized trypomastigotes stimulated over four times as much H_2O_2 as did nonopsonized T. cruzi. Interestingly, when amastigotes, the noninvasive intracellular form of T. cruzi was tested, H_2O_2 production did not correlate well with the percentage of macrophages associated with parasites (correlation coefficient = 0.535, Figure 1). Also, amastigotes stimulated lower amounts of H_2O_2 production at a given level of association than trypomastigotes (Figure 1). This was true even though high numbers of amastigotes had associated with the macrophages. Thus, the ability of the parasite to activate production of H_2O_2 by resident macrophages depended upon the form of the parasite.

Furthermore, a good correlation was also observed between the number of trypomastigotes per 100 macrophages and the amount of H_2O_2 production (Figure 2). The relationship was linear from 0 to approximately 80 parasites per 100 macrophages, at which point the amount of H_2O_2 produced reached a plateau (Figure 2). Thus, macrophages were capable of producing more H_2O_2 to presumably destroy the greater number of trypomastigotes with it.

One would expect that with an increase in H_2O_2 production more parasites would be destroyed if H_2O_2 was truly critical for killing. Indeed, using data from several experiments, it was found that, generally, there was a direct relationship between the amount of

killing of trypomastigotes, amatigotes, and opsonized trypomastigotes after 19-24 hours and the amount of H_2O_2 production (Figures 3 and 4). For trypomastigotes and amastigotes, approximately the same amount of H_2O_2 (15 nmoles/ mg macrophage protein) caused a 50% decrease in number of parasites per 100 macrophages (Figure 3). Approximately 3.5 times that amount of H_2O_2 coincided with a 50% decrease in number of opsonized trypomastigotes associated per 100 macrophages (Figure 4). For destruction of 25% of the parasites, 5 nmoles H_2O_2/mg protein were produced by trypomastigotes and amastigote-stimulated macrophages whereas opsonized trypomastigotes stimulated production of 50 nmoles H_2O_2/mg macrophage protèin. An increase in killing of T. cruzi with an increase in H_2O_2 production by macrophages agrees with previous literature that suggests that H_2O_2 production is important in destruction of T. cruzi (1, 2, 3, 37) since killing of T. cruzi was abrogated by scavengers of H_2O_2 (2, 3). However, this is the first instance where T. cruzi-stimulated H_2O_2 has been quantitated, correlated with degree of T. cruzi-macrophage association, or correlated with degree of killing of T. cruzi by macrophages.

The characteristics and degree of infectivity of the parasite are highly variable. This may be related to the progression of the parasites through each form in its life cycle. In a subsequent study which had much higher levels of association (260-900 T. cruzi trypomastigotes/100 macrophages), H_2O_2 production again correlated with the number of T. cruzi/100 macrophages (correlation coeffecient = 0.961, Figure 5). At these high levels of association, 85-100% of

Figure 3. Correlation between H_2O_2 production and killing of trypomastigotes or amastigotes. For measurement of H_2O_2 production, T. cruzi trypomastigotes were incubated for 2 hours with adherent macrophages in the reaction solution. Parasites were incubated for 2 hours with macrophages in DMEM and then the number of T. cruzi associated with macrophages were determined. The number of parasites associated with the macrophage at 2 hours was compared to the number still associated 19-24 hours later to determine killing of T. cruzi by macrophages. The points were from experiments performed on several days with freshly prepared macrophages, parasites, and reaction solution.

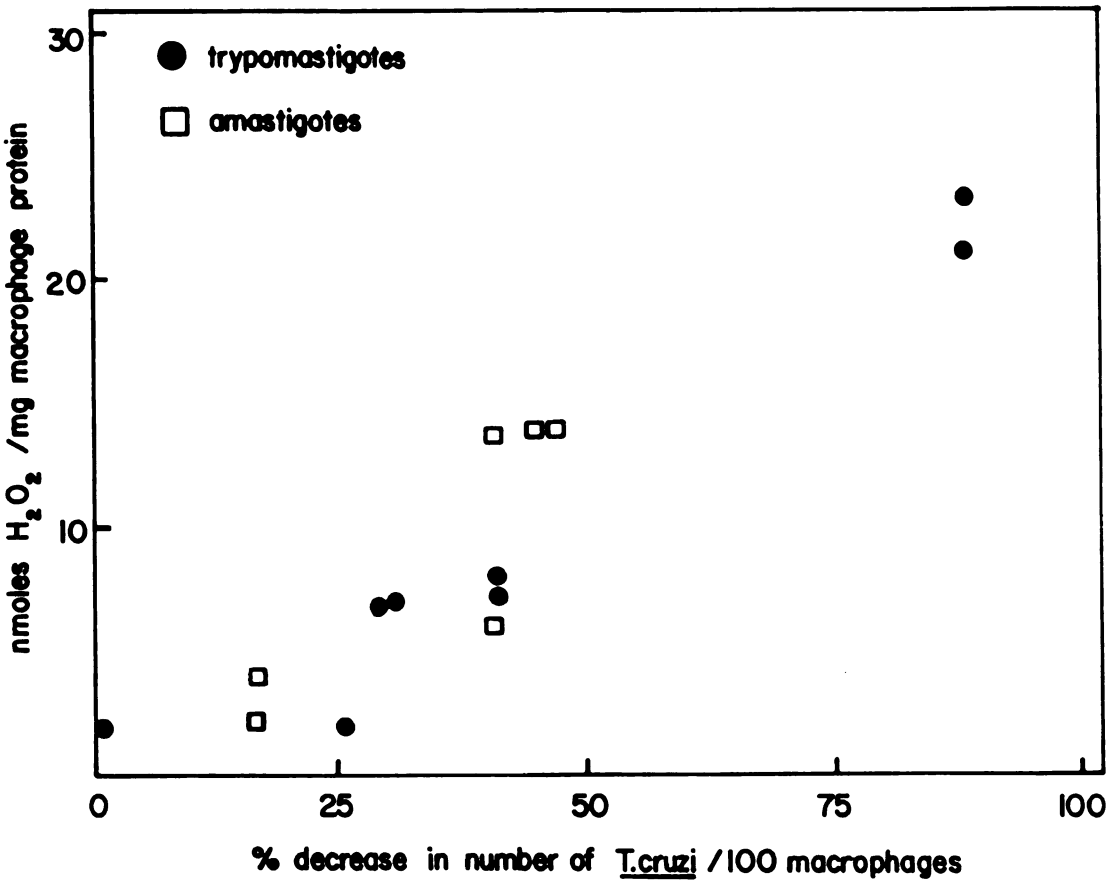

trypomastigotes
amastigotes
nmoles H_2O_2 /mg macrophage protein
30
20
10
0
25
50
75
100
% decrease in number of T.cruzi /100 macrophages

Figure 4. Correlation between H_2O_2 production and killing of opsonized trypomastigotes. For measurement of H_2O_2 production, T. cruzi trypomastigotes were incubated for 2 hours with adherent macrophages in the reaction solution. Parasites were incubated for 2 hours with macrophages in DMEM and then the number of T. cruzi associated with macrophages was determined. The number of parasites associated with the macrophage at 2 hours was compared to the number still associated 19-24 hours later to determine killing of T. cruzi by macrophages. The points were from experiments performed on several days with freshly prepared macrophages, parasites, and reaction solution.

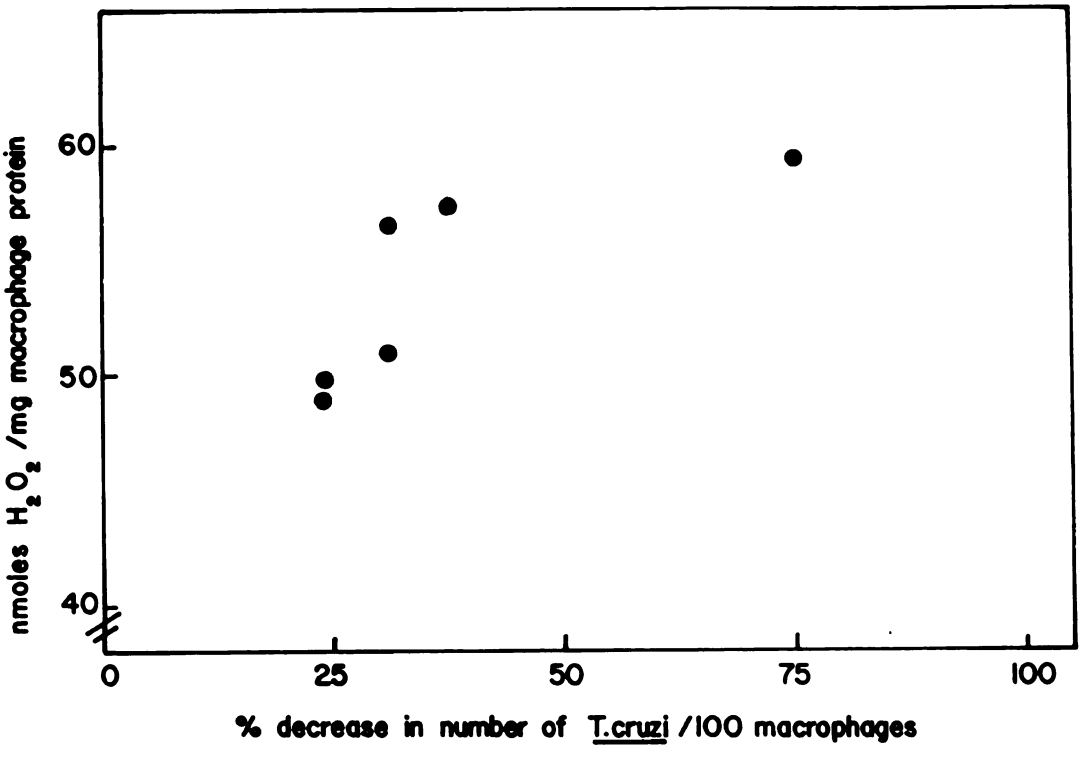
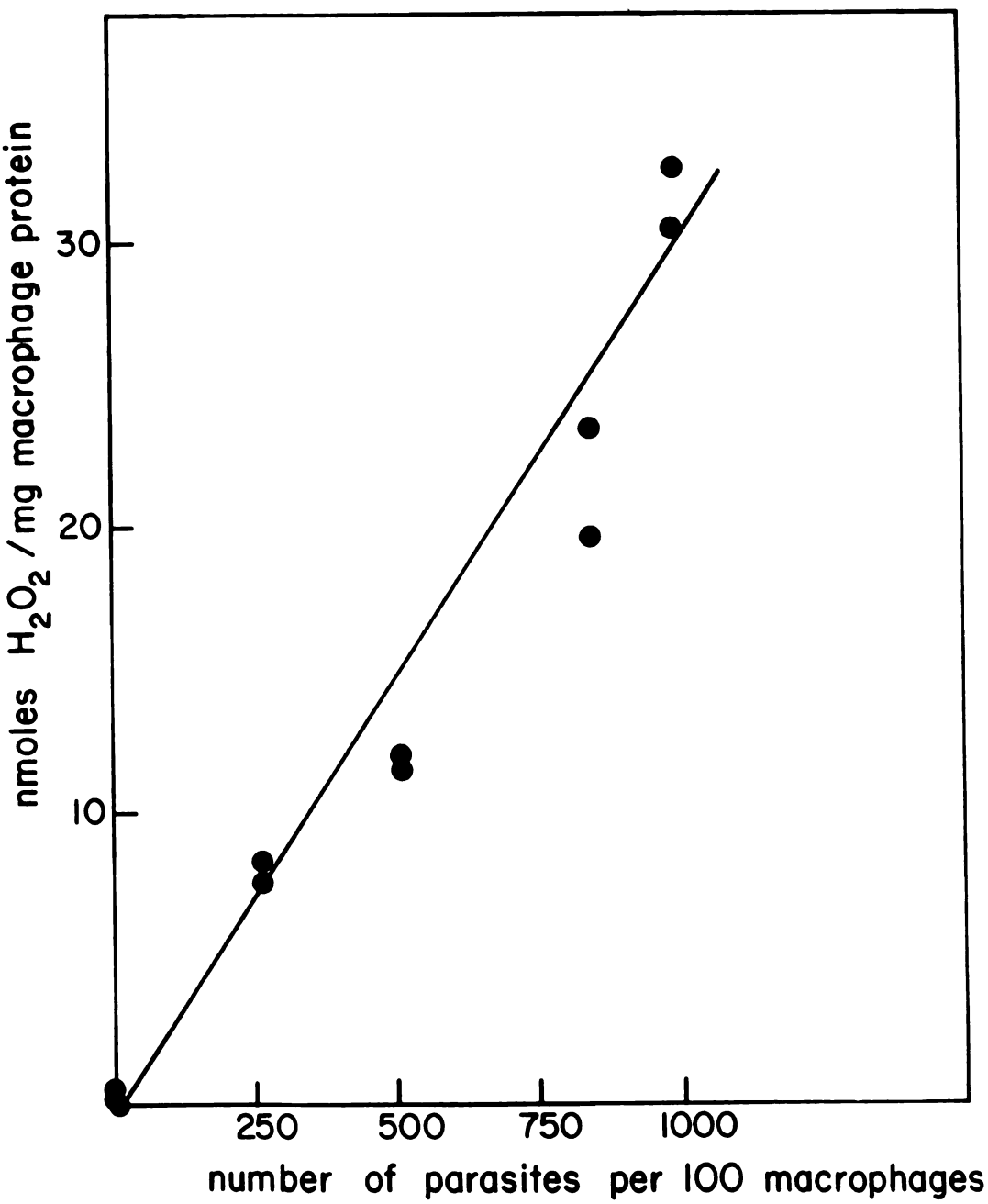

nmoles H_2O_2 /mg macrophage protein
60
50
40
0
25
50
75
100
% decrease in number of T.cruzi /100 macrophages

Figure 5. Correlation between H_2O_2 production and number of T. cruzi per 100 macrophages at high levels of T. cruzi-macrophage association. For measurement of H_2O_2 production, trypomastigotes were incubated for 2 hours with adherent macrophages in reaction solution. For the determination of the number of T. cruzi associated with the macrophages, parasites were incubated with macrophages in DMEM. Points are from a single assay.


nmoles H_2O_2 / mg macrophage protein
30
20
10
250
500
750
1000
number of parasites per 100 macrophages

the macrophages had associated with T. cruzi (data not shown). Therefore, for this set of data, the percentage of macrophages associated with parasites had reached a maximum degree of infectivity; thus, correlations between percentage of macrophages associated with T. cruzi and H_2O_2 production could not be done. At these high levels of association where significant amounts of H_2O_2 are produced, macrophage membrane integrity is compromised and the cells died within 24 hours (data not shown). Thus, correlations between H_2O_2 production and killing of the parasite could not be done for this set of data. Although, in Figure 2 and 5, the amount of H_2O_2 production, limits for maximal H_2O_2 production, and degree of T. cruzi-macrophage association were quite different, the amount of H_2O_2 consistantly correlated with T. cruzi-macrophage association. The observed variations in degree of association of T. cruzi with the macrophages are probably due to variablities in the ability of the parasite to infect and activate the macrophage.

The mechanism(s) for stimulation of H_2O_2 production by T. cruzi were unknown. However, one pathway by which T. cruzi could stimulate H_2O_2 production was analyzed. It was hypothesized that 20:4 or its metabolites may be involved in T. cruzi-stimulated H_2O_2 production since 20:4 metabolites are known to be stimulatory for the "oxygen burst" (12, 39) and, most importantly, exogeneous leukotrienes, an 20:4 metabolite, have been shown to increase T. cruzi association and killing (39, 40). The following preliminary data suggests that 20:4 metabolites may also be second messengers in T. cruzi-stimulated H_2O_2 production by resident macrophages. First, the amount of

endogenous 20:4 naturally present in macrophage phospholipids as a source for release of 20:4 was determined. Figure 6 shows that macrophage phospholipids contain a considerable amount of 20:4 (71 nmoles/mg macrophage protein); 21% of the fatty acid was 20:4 which is very close to the reported 25% (41). Next, release of 20:4 and its metabolites by macrophages stimulated with T. cruzi was investigated. Table 1 shows that incubation of T. cruzi trypomastigotes with resident macrophages stimulated the release of the arachidonic acid (20:4) metabolites, PGE_2, HETE's, and what may be leukotrienes, from macrophages in a ratio of 2:1:4. It has been shown by Fels et. al. (34) that the opsonized zymosan-stimulated release of radioactivity from ^{3}H-20:4 labeled macrophages is 20:4 and its metabolites. Further analysis by high pressure liquid chromatography of the radioactive peak comigratory with the leukotrienes would be necessary to confirm identification of the radioactive peak as leukotrienes. If these are identified as leukotrienes, one would want to try to correlate leukotriene production with infection and killing of T. cruzi. Furthermore, the amount of nonmetabolized 20:4 that was released upon stimulation of macrophages with T. cruzi was not greater than the spontaneous release of nonmetabolized 20:4 by nonstimulated macrophages (Table 1). Thus, T. cruzi trypomastigotes did stimulate the release of 20:4 but all of it was metabolized to PGE, HETE's and perhaps leukotrienes. Assuming that leukotrienes were produced, most (71%) of the 20:4 was metabolized via the lipoxygenase pathway and 29% went via the cyclo-oxygenase pathway. One may assume that the 20:4 released was derived from the phospholipids since others have

Figure 6. Fatty acid quantites in phospholipids from resident macrophages. Adherent macrophages were scraped into 0.15 M NaCl. Margaric phosphotidlycholine was added as an internal standard. Lipids were extracted by the Bligh and Dyer method. Phospholipids were separated from neutral lipids using a silicic acid column. Then, the fatty acids were transmethylated and quantitated by gas liquid chromatography techniques. The nanomoles of each fatty acid were calculated using the peak area of the internal standard. Protein content of the scraped cells was determined by the Lowry method.

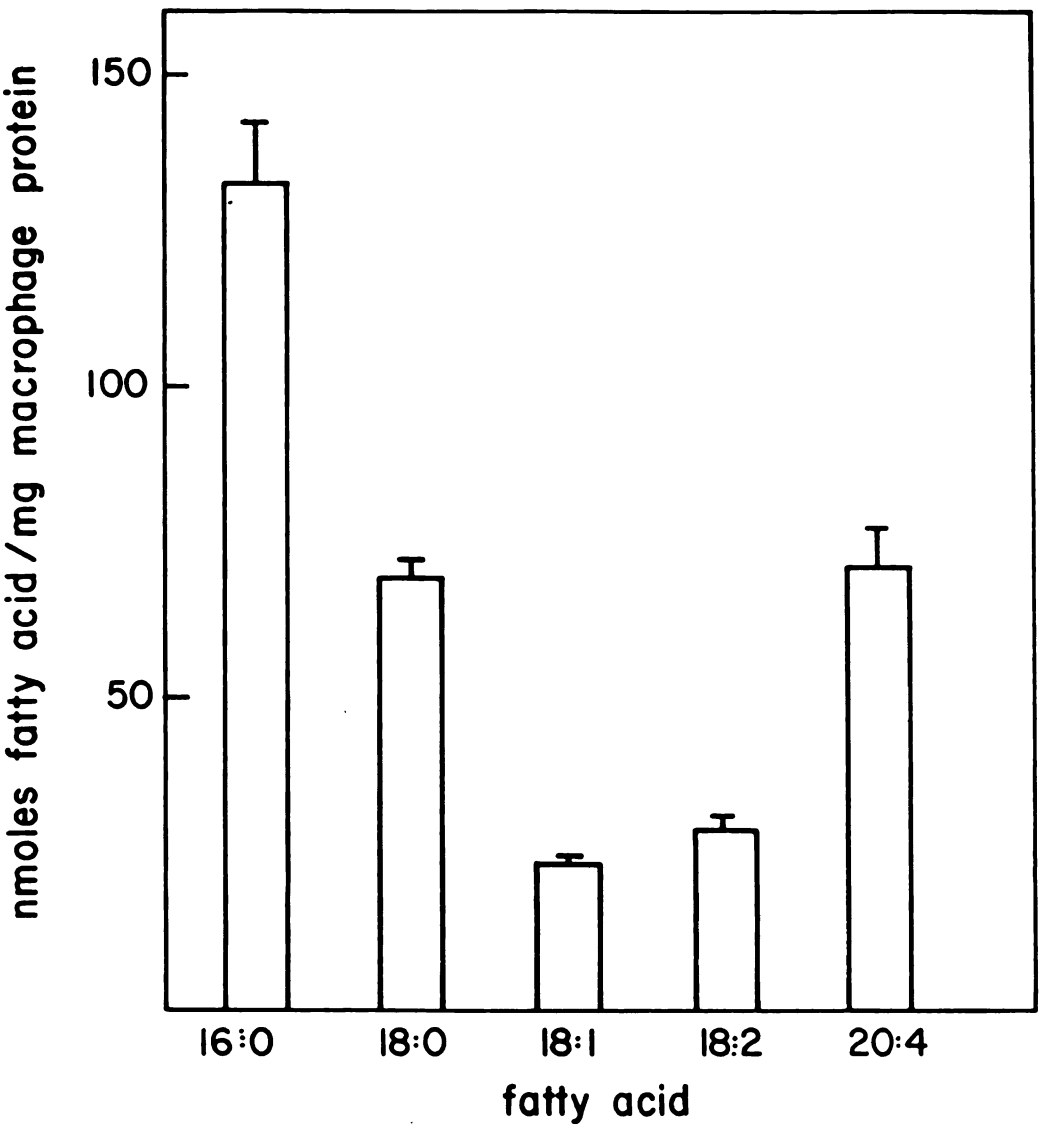

150
100
50
nmoles fatty acid/mg macrophage protein
16:0
18:0
18:1
18:2
20:4
fatty acid

TABLE 1

Release of ^{3}H-arachidonate Metabolites[a]

Rf[b]	Comigratory Standards	Stimulated Release ^{3}H (cpm)[c]	Spontaneous Release ^{3}H (cpm)[d]
0.02	LTC_4, LTD_4, LTE^4	302 ± 48	66 ± 12
0.35	PGE_2	150 ± 14	67 ± 7
0.67	HETE's	84 ± 48	205 ± 27
0.88	arachidonate	-11 ± 9	90 ± 14

[a] ^{3}H-arachidonate metabolites in supernatants from resident macrophages stimulated with T. cruzi at a T. cruzi:macrophage ratio of 25:1

[b] Rf of standards: 0.02 = (LTC_4, LTD_2, LTE_4), 0.28 = TxB_2, 0.32 = 6-keto PGF_2, 0.35 = PGE_2, 0.52 = LTB_4, 0.67 = HETE's, 0.79 = ricinoleic acid, 0.88 = arachidonate

[c] mean ± SEM for ^{3}H cpm released by stimulated macrophages minus ^{3}H cpm of spontaneous release by nonstimulated macrophages for each radioactive peak.

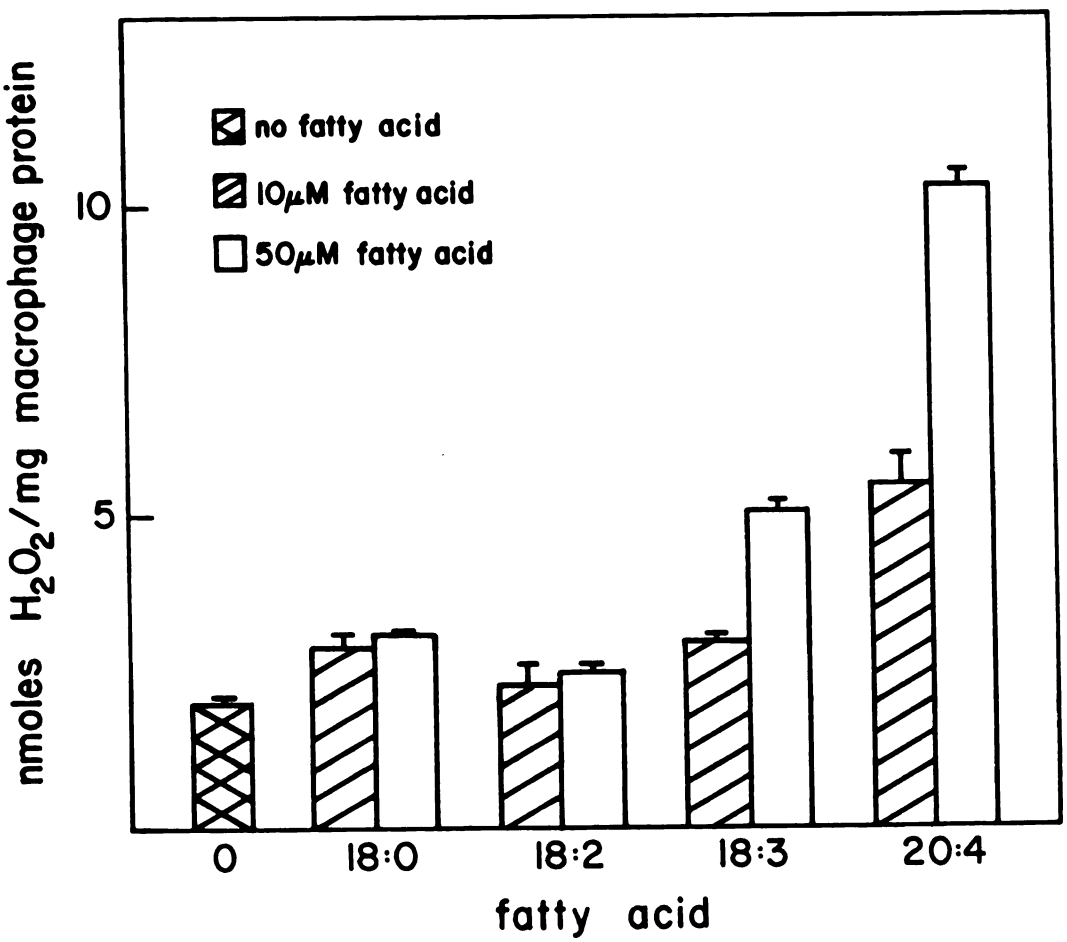
[d] ^{3}H cpm released by nonstimulated macrophages in the area coinciding with a stimulated radioactive peak

reported that the majority of the ^{3}H-20:4 is incorporated into the macrophage phospholipids (34). In this study, the macrophage phospholipids at an Rf of 0.94 on thin layer chromatography contained 49256 ± 9912 cpm of ^{3}H. Thus, the amount of radioactivity released (about 600 cpm ^{3}H) was small (1.2%) compared to the amount of ^{3}H incorporated into the phospholipids. Other stimulants such as opsonized zymosan have been shown to stimulate release of 6% to 15% of the ^{3}H that was incorporated into phospholipids of resident macrophages (36). Thus, T. cruzi may stimulate the release of smaller amounts of 20:4 than opsonized zymosan or phorbol. Also, T. cruzi may be less stimulatory than opsonized zymosan or phorbol since in Chapter 3 it was shown that T. cruzi was less stimulatory for H_2O_2 production than either opsonized zymosan or phorbol. Another possibility is that the assay conditions may need to be optimized. Also, since the data suggested that trypomastigotes may stimulate release of 20:4 from phospholipids to stimulate the "oxygen burst", the question comes to mind, "Are other fatty acids also involved in the stimulation of the oxygen burst by T. cruzi?" This was doubtful since fatty acids of shorter chain length and higher degrees of saturation than 20:4 have been reported to stimulate production of much smaller amounts of H_2O_2 than 20:4 (42, 43). To confirm that this relationship among the fatty acids for stimulation of H_2O_2 is true in our system with resident peritoneal macrophages from A/J mice, several fatty acids found in macrophage membranes were used to stimulate H_2O_2 production.

Figure 7 shows that several exogeneously added fatty acids did stimulate H_2O_2 production by resident peritoneal macrophages from A/J mice. Also, as reported previously (42, 43), the amount of H_2O_2 produced was dependent upon chain length and degree of saturation; 20:4 stimulated twice as much H_2O_2 production as 18:3, 18:2, or 18:0. Therefore, if T. cruzi stimulates release of fatty acids of shorter chain length or more saturation than 20:4, these shorter chain more-saturated fatty acids would probably not be as important in the oxygen burst stimulated by T. cruzi, since they were less stimulatory for H_2O_2 production than 20:4 and perhaps 20:4 metabolites. In summary, these preliminary results suggested that 20:4 metabolites may be second messengers in H_2O_2 production by macrophages stimulated with T. cruzi.

Another conceivable approach to showing that 20:4 metabolites are intermediates in T. cruzi-stimulated H_2O_2 production would be to inhibit lipoxygenase and/or cyclo-oxygenase activity of the macrophages. If this reduced 20:4 metabolite release as well as H_2O_2 production, it would suggest that 20:4 metabolites are involved in H_2O_2 production. However, although the inhibitors of lipoxygenase and cyclo-oxygenase are supposedly specific for lipoxygenase versus cyclo-oxygenase, these inhibitors alter many other cellular activities such as glucose metabolism (44, 45). Thus, this approach is dependent upon the development of a specific inhibitor of lipoxygenase or cyclo-oxygenase which does not affect other cellular processes.

Figure 7. H_2O_2 production by resident macrophages stimulated with exogeneous fatty acids. For determination of H_2O_2 production, exogeneous fatty acids were incubated with adherent macrophages for 2 hours in the reaction solution. Protein content was determined for macrophages incubated in DMEM without fatty acids.


no fatty acid
10μM fatty acid
50μM fatty acid
nmoles H_2O_2/mg macrophage protein
10
5
0
18:0
18:2
18:3
20:4
fatty acid

DISCUSSION

Although a considerable number of studies have been done to link H_2O_2 production by macrophages to destruction of T. cruzi, H_2O_2 production by macrophages stimulated with T. cruzi has not been quantitated. Using the modified HVA assay described in chapter 3, one may now measure T. cruzi stimulated H_2O_2 production by resident macrophages. In this chapter, the modified HVA assay was used to characterize T. cruzi stimulated H_2O_2 production. T. cruzi trypomastigotes that had been opsonized with chronic serum stimulated four times the amount of H_2O_2 production as nonopsonized trypomastigotes. Indeed, once the immune system mounts an antibody response to the parasite, trypomastigotes, the blood form of T. cruzi, may actually be opsonized in vivo. In comparison to the trypomastigotes, amastigotes, the noninvasive intracellular form of T. cruzi, stimulated half as much H_2O_2 production at a given level of association with the macrophages. Therefore, some forms of T. cruzi are more stimulatory than others for H_2O_2 production by resident macrophages. The mechanisms for association of the nonopsonized forms of T. cruzi are unknown. However, the opsonized trypomastigotes associate with the Fc receptors on the macrophages and are then phagocytosed (6, 7, 12, 13, 14, 15, 16). It has been shown that opsonization of T. cruzi facilitates uptake and destruction of the parasites by macrophages (46, 47). The enhanced destruction of opsonized parasites is probably related to the enhanced production of H_2O_2 by the macrophage. Still, until mechanisms for association of trypomastigotes

and amastigotes with macrophages are better defined, it is difficult to surmise a reason for why some T. cruzi forms are more stimulatory for H_2O_2 production than others at a given level of association.

It was also shown that H_2O_2 production by trypomastigotes and opsonized trypomastigotes but not amastigotes correlated well with the level of T. cruzi-macrophage association. Thus, there was a linear relationship between trypomastigote stimulation and H_2O_2 production. In addition, the maximum amount of H_2O_2 production by the macrophages varied with the batch of T. cruzi. Since from experiment to experiment the same number of T. cruzi incubated with macrophages will have varied degrees of association due to variations in infectivity of the parasites, correlations between H_2O_2 production and association are very important. The variations in infectivity by the parasite are probably related to the progression of the parasite through each of the forms in its life cycle.

Moreover, it was shown that H_2O_2 production is related to destruction of T. cruzi as suggested by others (1, 2, 3, 37), since as the amount of T. cruzi-stimulated H_2O_2 production by macrophages increased, the amount of killing of T. cruzi by macrophages also increased. The amount of H_2O_2 produced at 50% killing of trypomastigotes and amastigotes was the same. However, 3.5 times as much H_2O_2 was produced at 50% killing of opsonized trypomastigotes. This suggests that more H_2O_2 production may be required to kill opsonized trypomastigotes than trypomastigotes or amastigotes. However, since other factors besides H_2O_2 may be involved in the killing of T. cruzi, relative susceptibility of these forms of T. cruzi to H_2O_2 or

some H_2O_2 metabolite is still unknown. Still, there is one major problem with correlating H_2O_2 production to killing of T. cruzi. For detection of good quantities of H_2O_2, relatively high numbers of parasites must be associated with the macrophages and, at high levels of association, the parasites destroyed the macrophages at the 6 to 24 hours when killing of T. cruzi by macrophages is determined. Therefore, herein, at high levels of T. cruzi-macrophage association correlations could not be made between H_2O_2 production and killing of T. cruzi.

Also, in this chapter, the following possible mechanism for T. cruzi trypomastigote-stimulated H_2O_2 production was suggested. T. cruzi trypomastigotes stimulate release of the 20:4 metabolites, HETE's and what may be leukotrienes, which, in turn, stimulate NADPH oxidase located in the plasma membrane of the macrophage. NADPH oxidase catalyzes production of O_2^- which then dismutates to H_2O_2. In support of this series of events, preliminary data showed that trypomastigotes stimulated the release of mostly lipoxygenase products of 20:4; 71% of ^{3}H-cpm of stimulated release was HETE's and what may be leukotrienes. These results need to be verified by high pressure liquid chromatography before any sound conclusions can be made. Further support for the hypothesized mechanism is the fact that leukotrienes and HETE's are known to stimulate H_2O_2 production (12, 39). Moreover, T. cruzi did stimulate H_2O_2 production. The possibility that other fatty acids are also second messengers is doubtful since exogeneous addition of the fatty acids 18:0, 18:2, or 18:3 was much less stimulatory than 20:4. In fact, these results

agree with the literature which shows that, as the fatty acid chain length is shortened and the degree of fatty acid saturation is increased, there is less stimulation of H_2O_2 production by the fatty acid (42, 43). A considerable amount of endogeneous 20:4 was present as a possible source for release of 20:4 since 21% of the fatty acids in macrophage phospholipids was 20:4. This 21% is similar to the 25% reported in the literature for 20:4 in resident macrophages (41). 20:4 is also an intermediate in stimulation of NADPH oxidase by opsonized zymosan (6, 7, 12, 13, 14, 15, 16). However, whether opsonized zymosan and T. cruzi use the same receptors or mechanism for stimulation of the release of 20:4 from phospholipids remains to be determined. Opsonized zymosan enters the cell through receptor mediated phagocytosis. Unfortunately, the mechanism for T. cruzi invasion of macrophages and phagocytosis of T. cruzi by macrophages is unknown.

In summary, for the first time, T. cruzi has been used as the stimulant in the quantitation of H_2O_2 production. Also, this quantitation was done with resident macrophages instead of the activated macrophages. T. cruzi (trypomastigote and opsonized trypomastigote but not amastigote)-stimulated H_2O_2 production correlated with the level of parasite-macrophage association. Opsonization of trypomastigotes increased the amount of H_2O_2 produced. Amastigotes stimulated production of very low levels of H_2O_2 at a given level of association. Also, increased killing of trypomastigotes, opsonized trypomastigotes, and amastigotes seemed to be directly related to increased H_2O_2 production. Finally, the mechanism for T. cruzi trypomastigote

stimulated H_2O_2 production by macrophages probably involves 20:4 and its metabolites as second messengers. Future research may include verification of leukotriene production by T. cruzi-stimulated macrophages and, then perhaps, correlations between leukotriene production and T. cruzi-macrophage association or destruction of T. cruzi by macrophages. Also, in order to better understand T. cruzi infections, future research must include studies on variability in T. cruzi infectivity, mechanisms for association of T. cruzi with the macrophage, and the influence of factors made by other cell types on these processes as well as H_2O_2 production.

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Chapter 5

FUNCTIONAL CAPACITY OF RESIDENT PERITONEAL MACROPHAGES FROM ZINC DEFICIENT MICE

ABSTRACT

Zinc deficiency greatly increases the mortality rate of A/J mice infected with the parasite Trypanosoma cruzi (T. cruzi). This is in part due to the inability of resident macrophages from zinc deficient mice to phagocytose and destroy the parasite. Since destruction of T. cruzi is thought to be dependent on H_2O_2 production by the macrophage, we investigated the ability of resident peritoneal macrophages from zinc deficient mice to produce H_2O_2. Upon stimulation with the commonly used agents, phorbol 12-myristate 13-acetate (PMA, a chemical), or opsonized zymosan (a yeast cell extract), or arachidonate (the second messenger in opsonized zymosan stimulated H_2O_2 production), zinc deficient macrophages produced normal amounts of H_2O_2 as compared to zinc adequate macrophages. However, when T. cruzi was the stimulant, macrophages from severely and moderately zinc deficient mice produced lower amounts of H_2O_2 than the zinc adequate controls (66% and 83%, respectively, of controls; both of which are significantly different than control). However, when H_2O_2 production was expressed as amount of H_2O_2 per T. cruzi associated with the macrophages, similar quantities of H_2O_2 were produced by macrophages from all dietary groups. Thus, macrophages from zinc deficient mice could produce normal amounts of H_2O_2 upon stimulation with T. cruzi when the degree or number of associated parasites was compared to the amount of H_2O_2 production. This was not due to repair

in vitro since the amount of zinc in culture medium was always limiting (less than 1.5 μg/ dl).

The latter observations indicated that the failure of macrophages from zinc deficient mice to kill T. cruzi might be more related to reduced rate of association of the parasite with deficient macrophages. Association of T. cruzi trypomastigotes with macrophages involves both direct invasion by the parasite as well as macrophage phagocytosis. The receptors and mechanisms for association are unknown. However, the ability of macrophages to carry out phagocytosis has previously been found to be reduced if the content of long-chain saturated fatty acids in the phospholipids of the plasma membrane is increased. Also, previous studies have shown that livers from zinc deficient animals have an increased amount of long-chain saturated fatty acids. Thus, zinc deficient macrophages may also contain these fatty acids. However, when analyzed the amount of 16:0, 18:0, 18:1, 18:2, and 20:4 in the phospholipids, fatty acids were found to be the same for resident macrophages from zinc deficient and zinc sufficient mice.

Since exogeneous addition of leukotrienes has been shown to increase T. cruzi association and destruction, it remained to be determined which 20:4 metabolites were released upon stimulation with T. cruzi and if macrophages from deficient mice were capable of producing these metabolites. Before analyzing release of radiolabelled 20:4 metabolites, it was important to determine if macrophages from the deficient mice incorporated the same amount of radioactivity as controls. Indeed, macrophages from the zinc deficient

mice incorporated ^{3}H-arachidonic acid into the same types of phospholipids at equal quantities as macrophages from zinc adequate mice. Preliminary data indicated that, when stimulated with T. cruzi, macrophages from zinc deficient mice released three times as much of an arachidonic acid metabolite, comigratory with leukotrienes, as macrophages from zinc adequate mice. However, the release of the arachidonate metabolites PGE_2 and HETE by T. cruzi stimulated macrophages from deficient mice was normal as compared to macrophages from zinc adequate mice. Therefore, the increased amount of arachidonate released by macrophages from deficient mice compared to normal macrophages was metabolized to what may be leukotrienes. Before sound conclusions can be made concerning the radioactive peak comigratory with leukotrienes, the identity of this peak must be verified. However, it can be concluded that the decrease in association with and/or decreased destruction of T. cruzi by macrophages from deficient mice was not due to altered fatty acid composition of the macrophage phospholipids or reduced production of H_2O_2, respectively.

INTRODUCTION

Zinc deficiency increases the susceptibility of both humans and animals to disease and infections (1-6). Zinc deficient mice are more susceptible to infection with Trypanosoma cruzi (T. cruzi) (3), an obligate intracellular parasite which causes Chagas' disease in humans. Fraker et. al. (3) showed that there is a synergy between zinc deficiency and infection with T. cruzi. Upon infection, the mortality rate and the level of blood parasitemias were drastically increased in the zinc deficient mice as compared to infected zinc adequate controls or noninfected zinc deficient mice.

The inability of the mice to combat the T. cruzi infection was shown to be due, at least in part, to a reduced capacity of resident peritoneal macrophages to phagocytose and destroy the parasite (7, 8). In vitro, the percentage of macrophages which had T. cruzi bound to their surface and the number of T. cruzi per 100 macrophages were substantially reduced in the zinc deficient groups (7, 8). In addition, after 24 hours, macrophages from the zinc deficient groups were not able to destroy as many parasites as the zinc adequate group (7, 8). These defects were reversed by a half hour preincubation in vitro of the macrophages from zinc deficient mice with zinc at five times the physiological concentration (7, 8). Other metals such as copper and nickel were unable to restore parasite uptake and killing (7). Manganese had a slight restorative effect (7).

Therefore, the defect(s) in the zinc deficient macrophage seemed to be either directly or indirectly dependent on zinc.

Hydrogen peroxide produced by macrophages has been shown to correlate with destruction of T. cruzi (9-11). Also, addition of physiological levels of H_2O_2 has been shown to destroy T. cruzi (9, 10). Other oxygen metabolites such as OH^- and O_2^- are probably not involved in the destruction of T. cruzi since scavengers of these oxygen species do not affect the ability of the macrophages to kill T. cruzi (10). Furthermore, in chapter 4 of this thesis, it was shown directly that T. cruzi could indeed stimulate the production of quantifiable amounts of H_2O_2. However, taken together, this does not indicate that H_2O_2 is the afferent molecule that destroys T. cruzi. In fact, T. cruzi itself contains superoxide dismutase, the O_2^- scavenger, (12) but not catalase, the H_2O_2 scavenger, (12-14). Still, H_2O_2 is involved in the destruction of T. cruzi by macrophages.

Since zinc deficient macrophages have a reduced capacity to destroy T. cruzi, and H_2O_2 is important in the destruction of this parasite, it was presumed that, in the absense of zinc, macrophages might have a reduced capacity to produce H_2O_2 since it was demonstrated in chapter one that zinc was important for many reactions associated with the production of H_2O_2. In this chapter, the ability of zinc deficient macrophages to produce H_2O_2 was analyzed. In all studies on zinc deficient macrophages, the amount of zinc available was less than 1.5μg/dl. Thus, the amount of zinc available for

restoration of function by macrophages from zinc deficient was minimal.

Because of the reduced ability of T. cruzi to associate with macrophages from zinc deficient mice, it was also possible that alterations in the composition of the membrane or secondary messenger system were created by the deficiency. A reduced amount of 20:4 has been found in phospholipids of zinc deficient rat livers (15-22). Further, decreases in unsaturated long chain fatty acids such as 20:4 have been shown to result in reduced phagocytosis (23, 24). Therefore, since zinc is required for synthesis of long chain unsaturated fatty acids such as arachidonic acid (20:4) and alterations in fatty acid composition affects phagocytosis, it was thought that the macrophages from zinc deficient mice may have an altered fatty acid composition within their phospholipids. To test the possibility of a reduction in long chain unsaturated fatty acids such as 20:4 in phospholipids of macrophages from zinc deficient mice, the fatty acid composition of macrophage phospholipids from zinc deficient and zinc sufficient mice were compared. Furthermore, exogeneous addition of leukotrienes, lipoxygenase products of 20:4, has been shown to increase association with and killing of T. cruzi by macrophages (25, 26). Therefore, production of 20:4 metabolites may be altered in macrophages from zinc deficient mice resulting in reduced association with and killing of T. cruzi by deficient macrophages. Preliminary data in Chapter 4 indicated that T. cruzi stimulated the release of HETE's, PGE's and another 20:4 metabolite comigratory with leukotrienes by macrophages from normal mice.

Therefore, the relative amounts of these 20:4 metabolites released by the macrophages from zinc deficient and zinc adequate mice were compared. Also, to determine if any alterations among the dietary groups was due to altered release or incorporation of the ^{3}H-20:4, the amount of radioactivity incorporated into the macrophage phospholipids from the dietary groups was compared.

In summary, H_2O_2 production by macrophages and possible alterations in the membranes of macrophages from zinc deficient and zinc adequate mice was the subject of these studies.

MATERIALS AND METHODS

Animals. Female Crl-CD-1 (ICR)BR Swiss and female A/J mice were purchased from Jackson Laboratories, Bar Harbor, Maine. CD1 female mice were purchased from Charles Rivers, Portage, Michigan.

Diets. Six week old A/J female mice were placed in stainless steel cages with mesh bottoms to reduce recycling of zinc. They were fed ad libitum a biotin fortified egg white diet containing either deficient (0.8 μg Zn/g) or adequate (27 μg Zn/g) levels of zinc. The composition of the diet is described in Appendix. Since inanition accompanies zinc deficiency, a third group, the restricted mice, were fed zinc adequate diet equivalent to the average amount consumed the previous day by zinc deficient mice. All mice had free access to deionized distilled water (<0.2 μg Zn/g). Feed jars and water bottles were washed with 4N HCl and rinsed with deionized water to remove zinc. The mice were weighed at least once a week. At the end of the dietary period, those mice that received the zinc deficient diet and weighed 65-68% of the average body weight of the control mice were designated as severely zinc deficient mice. Moderately deficient mice were defined as weighing 70-74% of the average control mouse body weight. Previous studies indicated the latter group is only modestly effected by inanition (27, chapter 2).

Zinc analysis. The diets, culture media and sera were analyzed for zinc content by atomic absorption spectrophotometry (Varian

Techron AA-175, Springvale, Australia) as described in earlier publications (28, 29).

Isolation of Trypanosoma cruzi (T. cruzi). Four week old Crl-CD-1(ICR)BR Swiss mice were infected intraperitoneally with 2 x 10^5 blood forms (trypomastigotes) of T. cruzi. Twelve to 14 days later, blood was collected from the axillary artery of mice anesthetized with ether. Blood was collected in tubes containing heparin or disodium ethylene-diaminotetracetate (EDTA) powder so that the final concentrations were 25 U/ml or 2.5 mg/ml, respectively. Parasites from the blood were separated on a lympholite gradient (30) and passed through a diethylaminoethylcellulose column (31). The parasites were centrifuged (800 x G, 15 minutes, 4°C) and resuspended in Dulbecco's modified minimal essential medium (DMEM, Gibco, Grand Island, NY) supplemented with 100 IU penicillin and 100 µg streptomycin / ml. Greater than 99% of the cells were viable parasites in the trypomastigote form. Before use in the assays for H_2O_2, the parasites were washed two times in phosphate buffered saline (PBS, pH 7.4) containing 1% glucose and resuspended in the reaction solution for the H_2O_2 assay.

Preparation of opsonized zymosan. One mg of zymosan, a yeast cell extract (Sigma, St. Louis, MO), was incubated with one milliliter of complement inactivated mouse serum from A/J mice for 1 hour at 37°C. The opsonized zymosan was washed 2 times and resuspended in 10 mM PBS (pH 7.4). The zinc content of opsonized zymosan was 55 µg Zn/ g zymosan.

Collection, isolation, and identification of peritoneal macrophages. Cells were harvested from the unelicited mouse peritoneum by lavage with 5 ml of cold (4°C) DMEM and 0.5% w/v gamma globulin free bovine serum albumin (BSA, 0.4 μg Zn/ g BSA, Calbiochem, La Jolla, CA) (32). Cells were adhered to tissue culture plates for 1 1/2 hours at 37°C and 7% CO_2-ambient air. Nonadherent cells were removed by washing with 37°C DMEM. The percentage of macrophages-/monocytes adhered to the culture plate was determined by staining for nonspecific esterase activity as detailed in the modification (33) of the technique described by Yam et. al. (34). Always, 94-100% of the adherent cells were identified as monocytes/macrophages by staining for nonspecific esterase and by morphology.

Macrophage viability. Viability of the adherent macrophages was determined using the trypan blue exclusion method at the end of each assay. Always >95% of the macrophages were viable.

Association of T. cruzi with macrophages. After incubating the macrophages with parasites for 90 minutes in DMEM (<1 μg Zn/ dl) at 37°C and 7% CO_2-ambient air, the nonassociated parasites were removed by washing three times with PBS - 1% glucose. The cultures were incubated in DMEM for another 18 hours and/or fixed with methanol for 5 minutes, allowed to dry, and stained for 1 hour with Giemsa in 10 mM phosphate buffer (pH 6.8). The cells were washed one time with 10 mM phosphate buffer (pH 6.8). Numbers of parasites per 100 macrophages and percent of macrophages associated with parasites were determined by counting at least 200 cells per replicate.

Phenol Red Assay. The assay conditions described by Pick and Mizel (35) were modified for use with resident macrophages. Resident peritoneal exudate cells (7×10^5) in 100 µl were allowed to adhere to a 96 well flat bottom plate (Corning Glass Works, Corning, NY) for 1 1/2 hours. After removing nonadherent cells, the adherent macrophages were washed with 10 mM phosphate buffered saline (PBS) - 1% glucose (37°C). Forty microliters of the reaction solution consisting of 140 mM NaCl, 0.5 mM $CaCl_2$, 10 mM potassium phosphate buffer (pH 7.4), 2 mM NaN_3, 5.5 mM glucose, 0.56 mM phenol red (United States Biochemical Corporation, Cleveland, Ohio), and 19 U/ml horseradish peroxidase (Sigma, St. Louis, MO) were added to each well. The reaction solution contained 1.4 µg Zn/ dl. NaN_3 was included since it inhibits catalase, the cytoplasmic scavenger of H_2O_2. Phorbol 12-myristate 13-acetate (PMA, Consolidated Chemicals, Midland, MI) was diluted in ethanol and added to the cells in a volume of 2 µl per well so that the ethanol concentration was less than 1%. Opsonized zymosan or PMA stimulated cells were incubated for 90 minutes at 37°C under a humidified atmosphere of ambient air. Standards consisted of wells containing reaction solution and 0-60 µM H_2O_2 but no cells. Nonspecific production of H_2O_2 was determined by including wells containing cells but no stimulant or stimulant but no cells. The reactions were stopped and protein dissolved by the addition of 2 µl of 10 N NaOH. The absorbance at 610 nm of pooled triplicates was determined using a microcuvette and a spectrophotometer (Gilford, Oberlin, Ohio). Macrophage protein was determined by the Lowry (36). The data were presented as nmoles H_2O_2/mg

macrophage protein from the following calculation: (μM H_2O_2 derived from the standard curve) X (1 liter/1000 ml) X (0.132 ml final volume of sample) X (1000 nmoles/1 μmole)/(mg macrophage protein determined by the Lowry) = nmoles of H_2O_2/mg macrophage protein.

Homovanillic acid assay for H_2O_2. The assay conditions described by Ruch et. al. (37) were modified for use with resident macrophages and the stimulant, T. cruzi trypomastigotes. Resident peritoneal cells (2×10^6) in 1 ml DMEM - 0.5% BSA (1.5 μg Zn/ dl) were allowed to adhere to a 24 well flat bottom plate (Corning). Nonadherent cells were removed from the adherent macrophage monolayer by washing three times with PBS - 1% glucose (37°C). Two hundred microliters of reaction solution which consisted of PBS, 1% glucose, 0.09 mM $CaCl_2$, 0.05 mM $MgCl_2$, 200 μM homovanillic acid, and 2 U/ml horseradish peroxidase (1.4 μg Zn/ dl) were added per well. Arachidonate was diluted in ethanol and 2 μl added per well so that less than 1% of the reaction solution was ethanol and the final arachidonate concentration was 50 μM. However, when parasites were the stimulant, they were resuspended in the 200 μl of reaction solution to be added to the macrophages. Standards consisted of wells containing reaction solution and 0-20 μM H_2O_2 but no cells. Nonspecific production of H_2O_2 was determined by including wells containing cells but no stimulant or stimulant but no cells. The plates were centrifuged at 50 x G for 3-4 minutes to increase parasite-macrophage interactions and incubated for 90 minutes in a humidified atmosphere at 37°C and 7% CO_2-ambient air. The reaction was stopped by the addition of 25 μl of 25 mM EDTA and 0.1 M glycine at pH 12. The relative fluorescence

of the homovanillic acid dimer was determined using a spectrofluorometer (Perken-Elmer 650-40) equipped with a microcuvette. The excitation and emission wavelengths were 312 nm and 420 nm, respectively. The slit widths for excitation and emsision were 2 nm and 5 nm, respectively. The amount of H_2O_2 in the samples was analyzed by linear regression of the standard curve. The amount of macrophage protein was determined by the method described by Lowry (36). The data are presented as nmoles H_2O_2 / mg macrophage protein from the following calculation: (μM H_2O_2 derived from the standard curve) X (1 liter/1000 ml) X (0.225 ml final volume of sample) X (1000 nmoles/1 μmole) / (mg macrophage protein as determined by the Lowry) = nmoles H_2O_2 / mg macrophage protein.

Synthesis of margaric phosphatidylcholine (38). Margaric (17:0) phosphatidylcholine was chosen as an internal standard for the analysis of fatty acid composition of macrophage phospholipids since methylated margaric acid has a different retention time during gas liquid chromatography than fatty acids normally found in good quantities in mammalian phospholipids. Margaric acid (200 mg) was placed in a small teflon capped tube with 300 μl thionyl chloride, pulsed with nitrogen, and placed on a steam bath for 1 hour. The products were evaporated to dryness under nitogen in a 80°C water bath, washed successively with water, 10% sodium carbonate, and water, and then dried under nitrogen. The fatty acyl chloride (50 mg) was reacted with the $CdCl_2$ complex of L-alpha-glycerophosphorylcholine (10.45 mg) while stirring for 2 hours in 150 μl chlorform and 9 μl pyridine under an atmosphere of nitrogen. The stirrer was rinsed with

chloroform and the solvents were evaporated under nitrogen. The reaction products were dissolved in 166 µl ether (dry), cleared by centrifugation, and the solvents removed under nitrogen. The lipids were dissolved in 500 µl acetone (dry), placed on a acetone-CO_2 bath for 1 hour and isolated by centrifugation in the cold. They were precipitated again with 250 µl acetone. The lipids were dried under nitrogen, dissolved in chloroform:methanol:water (5:4:1, v:v:v) and passed over a 2ml column of amberlites IR50:IRC50 (50: 50). The column was washed with the solvent mixture. The effluent was dried under nitrogen, dissolved in chloroform and placed over a 1 ml silicic acid column. The silicic acid column was washed with 30 column volumes of chloroform and the phospholipid was eluted with 15 column volumes of chloroform:methanol (1:1) followed by 10 column volumes of methanol. The eluent was evaporated under nitrogen and the phospholipid was stored dry under nitrogen at -20°C. The last 5ml fraction of the silicic acid column effluent did not contain margaric acid as measured by gas liquid chromatography of the fatty acid methyl ester. Esterification is described below. The phospholipid fraction from the silicic acid column contained margaric acid as determined by gas liquid chromatography of the methyl ester.

Fatty acid composition of macrophage phospholipids. Macrophages were adhered for 2 hours to 24 well tissue culture plates in DMEM-0.5% BSA (1.5 µg Zn/ dl). Nonadherent macrophages were removed by washing three times in DMEM-0.5% BSA. The adherent macrophages were collected by scraping into 0.15M NaCl. Since one twelfth of the amount of the above synthesized internal standard, margaric

phosphatidlycholine, gave a peak height during gas liquid chromatography that was 80% of the chart range, this amount of internal standard was added to each sample to control for sample loss during the purification of the macrophage phospholipids. The lipids were extracted by the method described by Bligh and Dyer (39). Briefly, methanol:chloroform:water (4:2:8, v:v:v) was added to the macrophages. Then one volume of chloroform followed by one volume of water was added to the extraction mixture. The mixture was centrifuged and the chloroform layer was collected and evaporated under nitrogen. The lipids were resuspended in chloroform and placed over a 0.5 ml heat activated silicic acid column in a pasteur pipette. The neutral lipids which do not bind to the column were washed through the column with chloroform. The phospholipids bound to the column were then eluted with chloroform:methanol (1:1) followed by methanol. The organic solvents were evaporated from the neutral and phospholipids under nitrogen. The fatty acids were converted to methyl esters (40) by incubating overnight at 75°C in 6% HCl in methanol under nitrogen. The fatty acid methyl esters were extracted with three equal volumes of hexane and washed once with water. Water was removed from the hexane by the addition of anhydrous sodium sulfate. The hexane was collected and evaporated under nitrogen. The fatty acid methyl esters were dissolved in hexane and analyzed (41) using a Hewlett-Packard gas chromatograph equipped with a 6 foot x 1/8 inch i.d. glass column packed with 10% SP-2330 Chromasorb W/AW. Analysis was performed isothermally at 180°C for 7 minutes followed by an increase of 4°C /minute to a limit of 200°C. The carrier gas-flow

rate was 30 ml/minute. Peak identifications were based on relative retention times of standard methyl esters (Nu Chek Prep, Elysian, Minnesota). Relative peak areas were measured by a Hewlett-Packard 3380A electronic integrator. Fatty acid quantities were obtained by linear regression from a standard curve of peak area versus amount of fatty acid methyl ester. Total macrophage fatty acid was determined by comparing peak area of the internal standard in the samples of macrophage phospholipids to peak area of a sample of internal standard alone that had not been submitted to the phospholipid isolation procedure. Also, samples of macrophage phospholipids from severely zinc deficient, moderately zinc deficient, restricted, and zinc adequate mice, which did not contain the internal standard, did not have a peak with the same retention time as the internal standard. Thus, since the macrophage phospholipids did not contain margaric acid, margaric acid was a valid internal standard.

Arachidonic acid release by macrophages (42, 43). Macrophages adhered to 24 well plates were incubated with 1.5 μCi (5, 6, 8, 9, 11, 12, 14, 15-^{3}H)arachidonic acid (Amersham) in 2 ml of DMEM overnight and washed three times with serum free DMEM (<1 μg Zn/ dl) at 37°C. The macrophages were stimulated by trypomastigotes of T. cruzi for 2 hours in serum free DMEM. Nonstimulated macrophages were included to control for spontaneous release of radioactivity by the macrophages. The culture medium was removed from the adherent macrophage monolayer, centrifuged, and acidified to 0.03M with citric acid. The acidified media was extracted three times with 3 ml of chloroform:methanol (2:1, v:v). The chloroform layers were combined,

washed two times with 2 ml of methanol:water (2:1, v:v) and evaporated to dryness under nitrogen. The residue was dissolved in 40 µl of ethylacetate:methanol (3:1, v:v) and applied to precoated plates (Silica Gel 60, Merck) for thin-layer chromatography. The internal standards PGE_2 and arachidonate were added to each sample. Other standards 6-keto-PGF_{1alpha}, LTB_4, LTC_4, LTD_4, LTE_4, and ricinoleic acid (an oxidized product of arachidonate) were run separately along with PGE_2 and arachidonate. Arachidonate and its metabolites were separated by development twice in the solvent system chloroform:ethylacetate:methanol:acetic acid:water (140:60:16:2:1, v:v:v:v:v). The internal standards were visualized by exposure to I_2. Half centimeter segments for each ascending sample of the silicic acid coated plates were scraped and radioactivity was measured by a Delta 300 scintillation counter (Tracor Analytic). Radioactivity for each segment of a sample was plotted versus distance (cm) on the plate. The data for each radioactive peak are presented as ^{3}H-cpm of stimulated release minus ^{3}H-cpm spontaneous release from nonstimulated samples.

^{3}H-arachidonic acid labelled macrophage phospholipids. The identification of those macrophage phospholipids that contained ^{3}H-arachidonate was described by Emilsson et. al. (44, 45). Briefly, radiolabelled resident macrophages were collected by scraping into 1 ml of ice-cold 10 mM HCl. Lipids were extracted with 6 ml of chloroform:methanol (1:1, v:v). Phase separation was obtained by addition of 2 ml of 10 mM HCl followed by centrifugation. The chloroform layer was removed, evaporated to dryness, and dissolved in 40 µl ethylacetate:methanol (3:1, v:v). The lipids were separated by thin

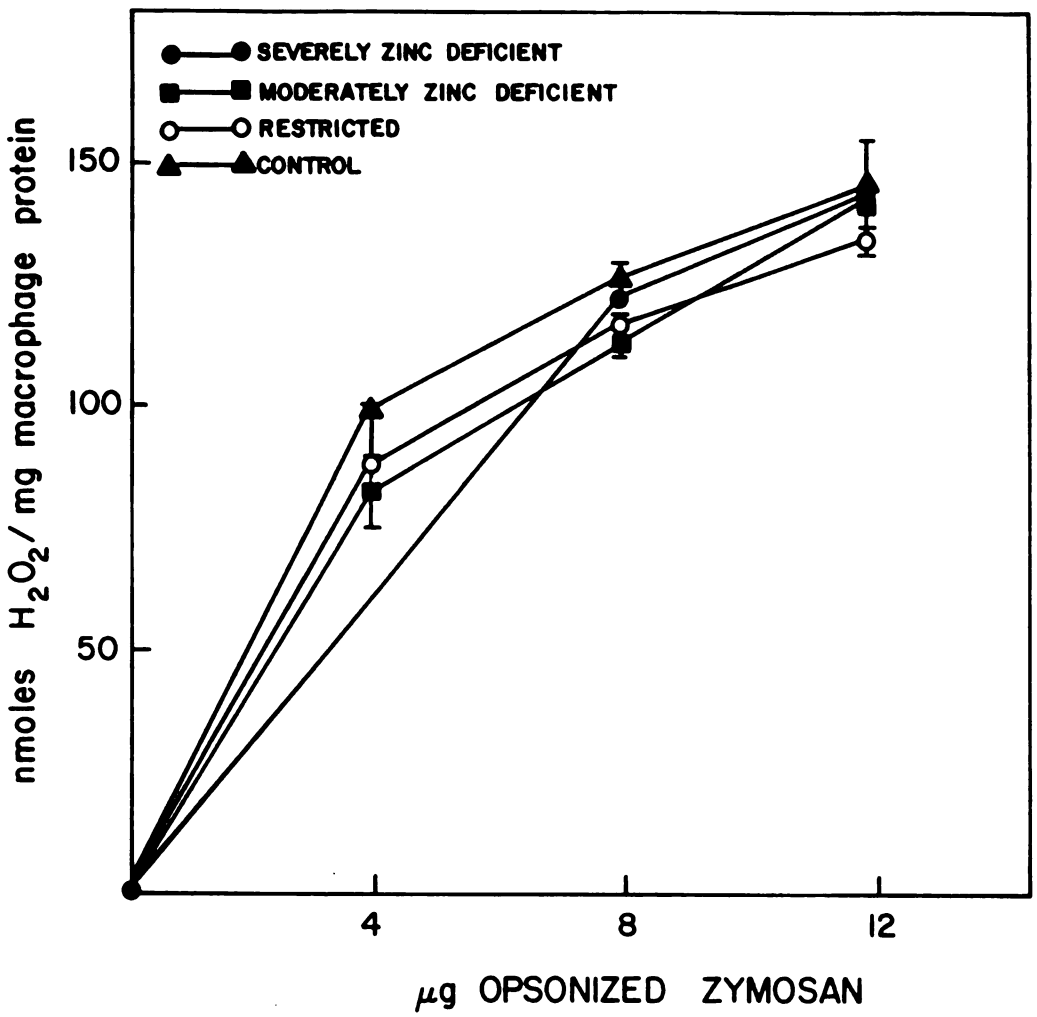
layer chromatography on precoated plates (Silica Gel 60, Merck) with the solvent system chloroform:methanol:acetic acid:water (25:20:3:0.3, v:v:v:v). Lipids were visualized by exposure to I_2. The plates were scraped in 0.5 cm segments for each ascending sample and radioactivity was measured by a Delta 300 scintillation counter (Tracor Analytic). Radioactivity for each segment of a sample was plotted versus distance on the plate. The total radioactivity for each peak was calculated.

Statistics. The mean and standard error of the mean were calculated for each treatment group. Probability values for the comparison of the zinc deficient and restricted groups to the control group were determined by a completely random Anova followed by Dunnett's Test.

RESULTS

We analyzed the ability of resident peritoneal macrophages from zinc deficient mice to produce H_2O_2 upon stimulation with several agents. The amount of zinc present during these studies was minimal since the medium for isolation of the macrophages contained <1 μg Zn/ dl and the reaction solutions for measuring H_2O_2 production contained less than <1 μg Zn/ dl. First, opsonized zymosan, a yeast cell extract to which autologous serum antibodies have been bound, or the second messenger in opsonized zymosan stimulation of H_2O_2 production, arachidonate (20:4), were exogeneously added to the macrophage monolayer to stimulate H_2O_2 production. If opsonized zymosan-stimulated H_2O_2 production by macrophages from zinc deficient mice was reduced, stimulation of the deficient macrophages with exogeneous 20:4 would help delineate whether a defect was in the release of the second messenger 20:4 or in the stimulation of the "oxygen burst" by 20:4. However, at several concentrations of opsonized zymosan, resident peritoneal macrophages from zinc deficient mice were capable of producing normal amounts of H_2O_2 over a period of 90 minutes when compared to macrophages from zinc adequate mice (Figure 1). At the highest dose of 12 μg of opsonized zymosan, 140 $\pm$ 10 nmoles H_2O_2 were produced per mg protein by macrophages from zinc deficient or zinc sufficient mice. Also, exogeneous addition of 20:4 (50 μM) stimulated production of the same quantities of H_2O_2

Figure 1. Opsonized zymosan-stimulated H_2O_2 production by resident peritoneal macrophages prepared from severly zinc deficient, moderately zinc deficient, restricted, or control mice as measured by the phenol red assay. Adherent macrophages from 2.1 x 10^6 peritoneal cells were incubated for 2 hours with 4, 8, or 12 μg opsonized zymosan in the reaction solution and H_2O_2 content determined. Protein content of macrophages incubated in DMEM for 2 hours was the same per well for all dietary groups. Each point represents the mean ± SEM for triplicates for pooled peritoneal cells from 10 to 15 mice.


SEVERELY ZINC DEFICIENT
MODERATELY ZINC DEFICIENT
RESTRICTED
CONTROL
150
100
50
nmoles H_2O_2/ mg macrophage protein
4
8
12
μg OPSONIZED ZYMOSAN

(about 9 nmoles H_2O_2 / mg macrophage protein) by macrophages from severe, moderate, restricted, and control mice (Figure 2). For these and all subsequent experiments, the amount of macrophage protein as determined by the Lowry was the same for all dietary groups (data not shown). Thus, the same number of macrophages had adhered to the plate for each dietary group and the same number of cells in each dietary group were responding in these experiment. Also, greater than 95% of the adherent cells for all dietary groups were viable and were identified as macrophages by staining for nonspecific esterase and by morphology. Therefore, the mechanism for opsonized zymosan-stimulated H_2O_2 production was unaltered in the macrophages from zinc deficient compared to zinc adequate mice.

Although opsonized zymosan is a more natural stimulant, phorbol 12-myristate 13-acetate (PMA), a chemical, seems to be the agent most often used to stimulate H_2O_2 production. Upon stimulation with PMA, macrophages from zinc deficient mice again produced normal amounts of H_2O_2 in 90 minutes as compared to H_2O_2 production by macrophages from zinc adequate mice (Figure 3). Therefore, H_2O_2 production and the mechanism for stimulation of H_2O_2 production by PMA was unaltered by the deficiency.

Since T. cruzi may have a different mechanism of entry into the macrophages than opsonized zymosan or PMA, it was important to measure H_2O_2 production by T. cruzi-stimulated macrophages from zinc deficient and zinc adequate mice. Using the improved assay conditions for measuring H_2O_2 production described in Chapter 3 and T. cruzi as the stimulant, the macrophages from severely zinc deficient mice produced

Figure 2. Arachidonate-stimulated H_2O_2 production by resident peritoneal macrophages prepared from severly zinc deficient, moderately zinc deficient, restricted, or control mice as measured by the HVA assay. Adherent macrophages from 2 x 10^6 peritoneal cells were incubated for 2 hours in the reaction solution with 50 μM arachidonic acid and H_2O_2 content determined. Protein content of macrophages incubated in DMEM for 2 hours was the same per well for all dietary groups. Each bar represents the mean ± SEM of triplicates of pooled peritoneal cells from 10 to 15 mice.

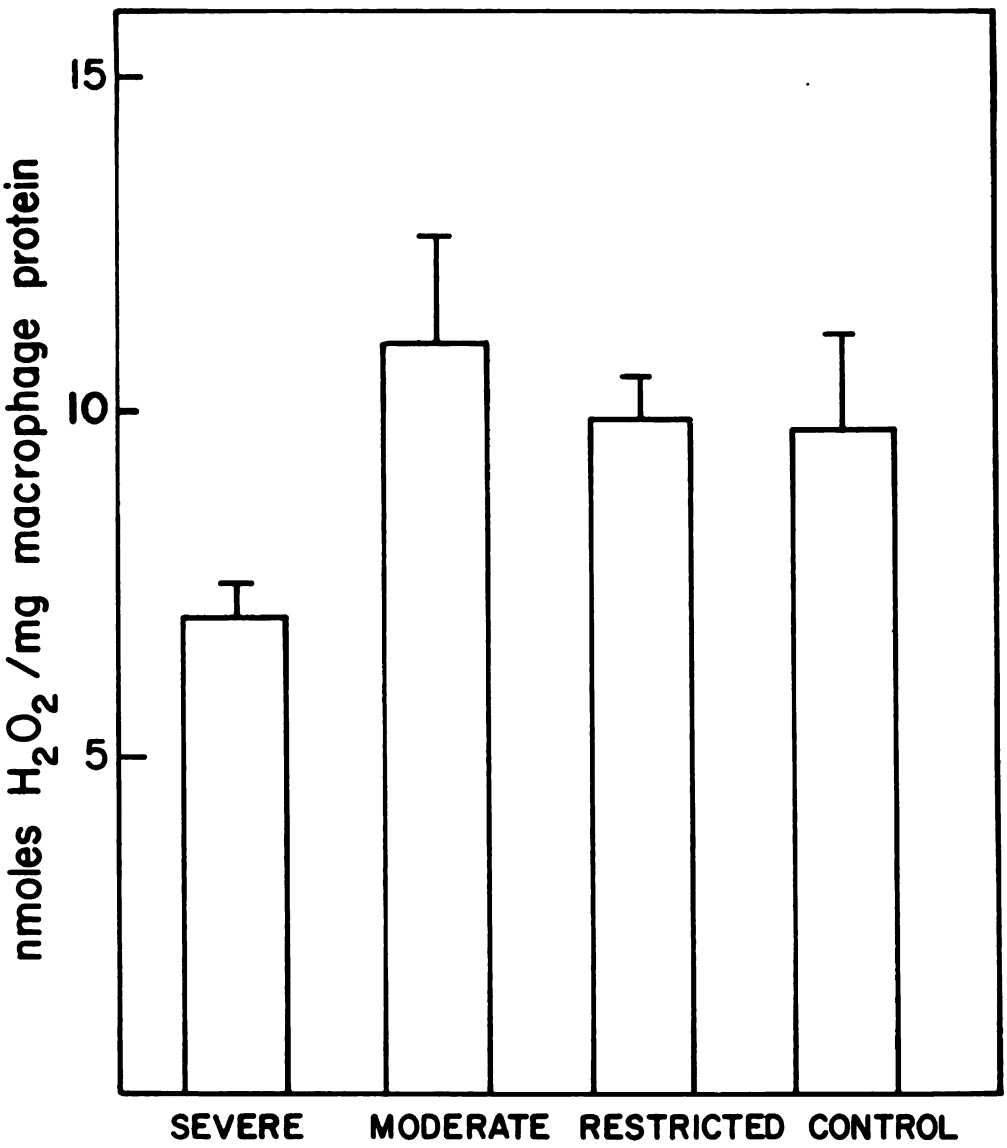
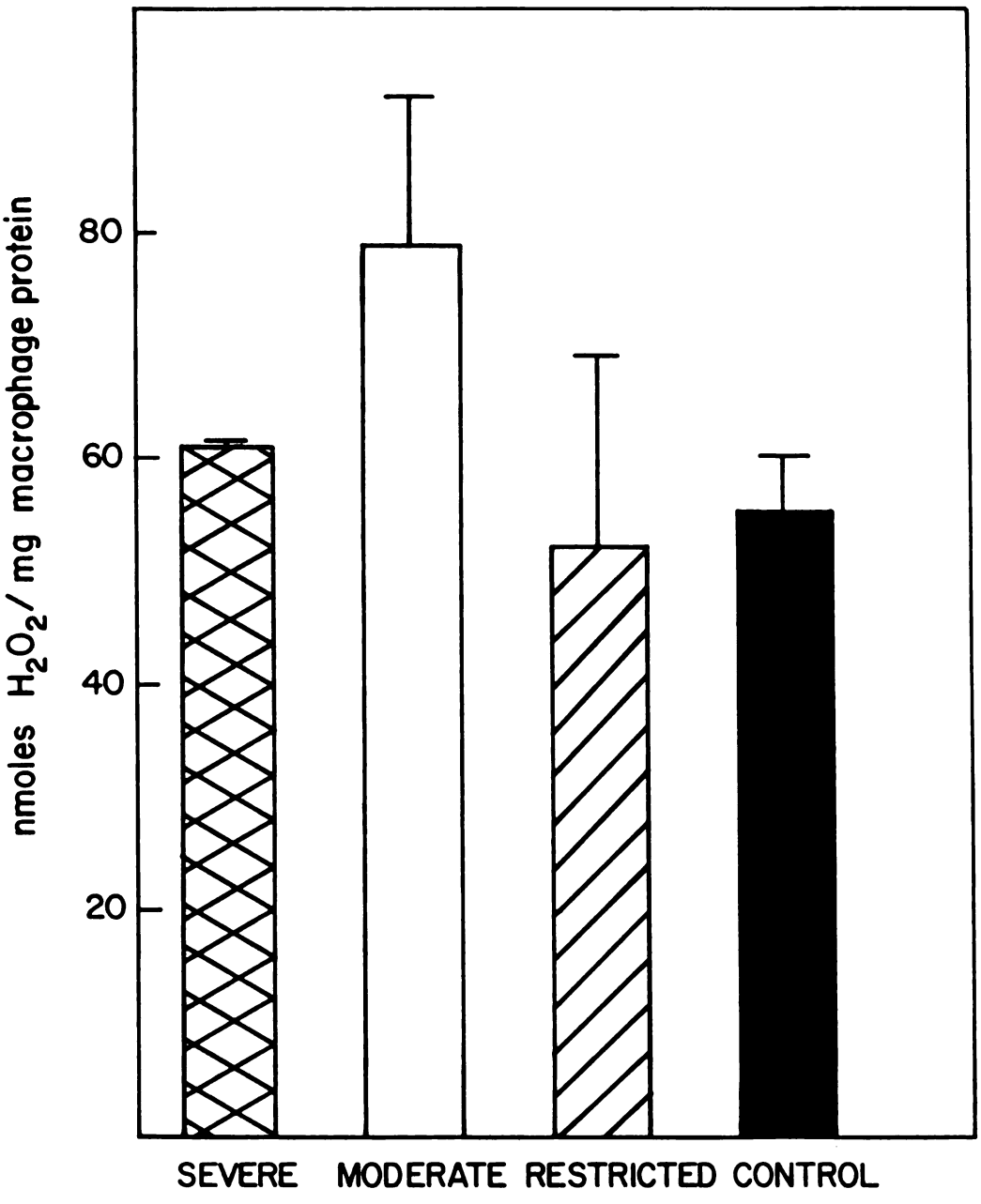

15
10
5
nmoles H_2O_2 /mg macrophage protein
SEVERE
MODERATE
RESTRICTED
CONTROL

Figure 3. PMA-stimulated H_2O_2 production by resident peritoneal macrophages prepared from severely zinc deficient, moderately zinc deficient, restricted, or control mice as determined by the phenol red assay. Adherent macrophages from 2.1 x 10^6 peritoneal cells were incubated for 2 hours with 3 μg PMA in the reaction solution and H_2O_2 content determined. Protein content of macrophages incubated in DMEM for 2 hours was the same per well for all dietary groups. Each bar represents the mean ± SEM of triplictes of pooled peritoneal cells from 10 to 15 mice.


nmoles H_2O_2/ mg macrophage protein
80
60
40
20
SEVERE
MODERATE
RESTRICTED
CONTROL

63 to 65% as much H_2O_2 as zinc adequate controls for the various T. cruzi:macrophage ratios tested (Figure 4). The H_2O_2 production by macrophages from moderately zinc deficient mice was also significantly lower than controls (65-73% of controls). However, the level of T. cruzi:macrophage association was too low for accurate quantitation of association since there was only one parasite per macrophage and only 10 $\pm$ 5% of the macrophages had parasites associated with them. Unfortunately, the degree of infectivity of this parasite is highly variable and unpredictable since not enough is known about the mechanism(s) of parasite-macrophage association. Quantitation of T. cruzi-macrophage association is very important for interpretation of the results, since in chapter 4 it was shown that H_2O_2 production correlates with T. cruzi-macrophage association. An interpretation of the reduced H_2O_2 production by T. cruzi-stimulated macrophages from zinc deficient mice can not be made without knowing the T. cruzi-macrophage association.

In a subsequent experiment, the parasites were much more infective and, again, H_2O_2 production by the macrophages from the deficient mice was significantly less than controls (Figure 5). In this case, at the optimal T. cruzi:macrophage ratio of 30:1, the severely and moderately zinc deficient groups were 66% and 83%, respectively, of controls. However, the number of T. cruzi associated with severely and moderately zinc deficient macrophages was also significantly reduced (71% and 75%, respectively, of controls at a T. cruzi:macrophage ratio of 30:1) (Figure 6). This reduction in the number of

Figure 4. Experiment 1: T. cruzi-stimulated H_2O_2 production by resident peritoneal macrophages prepared from severly zinc deficient, moderately zinc deficient, or control mice as measured by the HVA assay. Adherent macrophages from 2 x 10^6 peritoneal cells were incubated for 2 hours in the reaction solution with parasites at a T. cruzi:macrophage ratio of 20:1 or 35:1 and H_2O_2 content was determined. Protein content of macrophages incubated in DMEM for 2 hours was the same per well for all dietary groups. Each bar represents the mean $\pm$ SEM of triplicates from pooled peritoneal cells of 10 to 15 mice. Asterisk indicates significance of $p < 0.05$ or better as compared to the control group for the same T. cruzi:macrophage ratio.

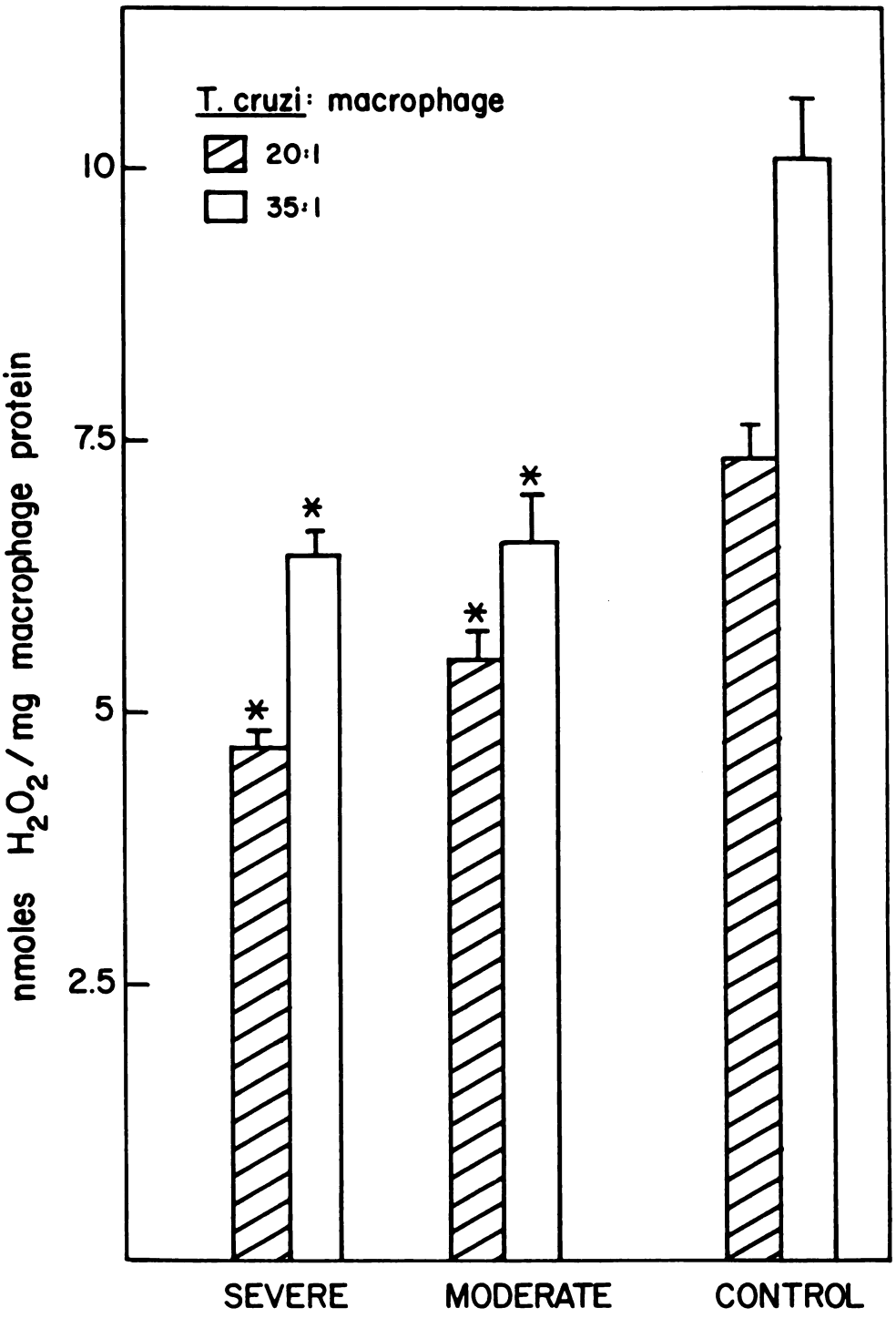

T. cruzi: macrophage
20:1
35:1
10
7.5
5
2.5
nmoles H_2O_2 / mg macrophage protein
*
*
*
*
SEVERE
MODERATE
CONTROL

Figure 5. Experiment 2: At high levels of T. cruzi-macrophage association, T. cruzi-stimulated H_2O_2 production by resident peritoneal macrophages prepared from severly zinc deficient, moderately zinc deficient, or control mice as measured by the HVA assay. Adherent macrophages from 2 x 10^6 peritoneal cells were incubated for 2 hours in the reaction solution with parasites at a T. cruzi:macrophage ratio of 5:1, 10:1, 20:1 or 30:1, and H_2O_2 content was determined. Protein content of macrophages incubated in DMEM for 2 hours was the same per well for all dietary groups. Each bar represents the mean $\pm$ SEM of triplicates from pooled peritoneal cells of 10 to 15 mice. Asterisk indicates significance of $p < 0.05$ or better as compared to the control group for the same T. cruzi:macrophage ratio.

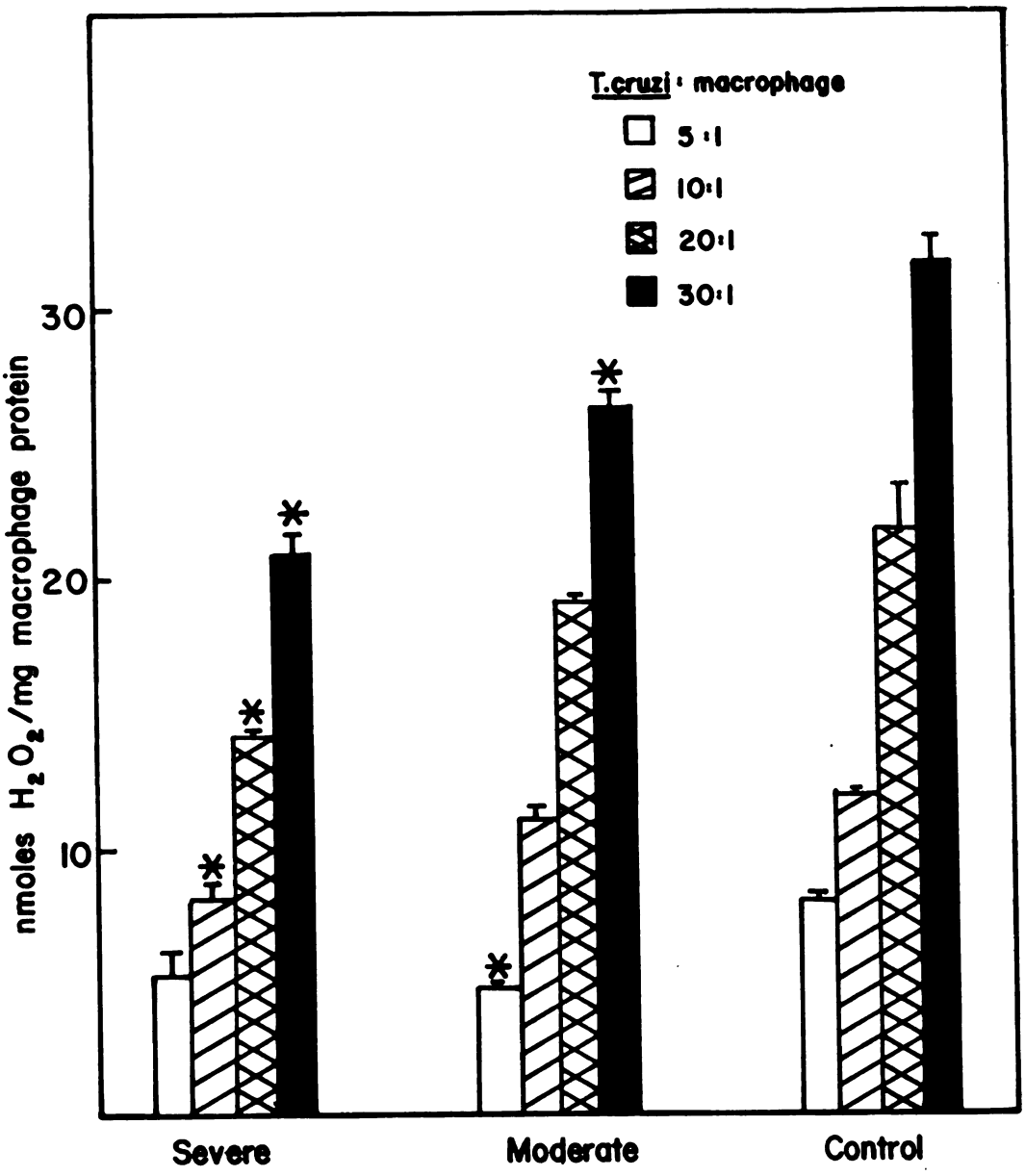
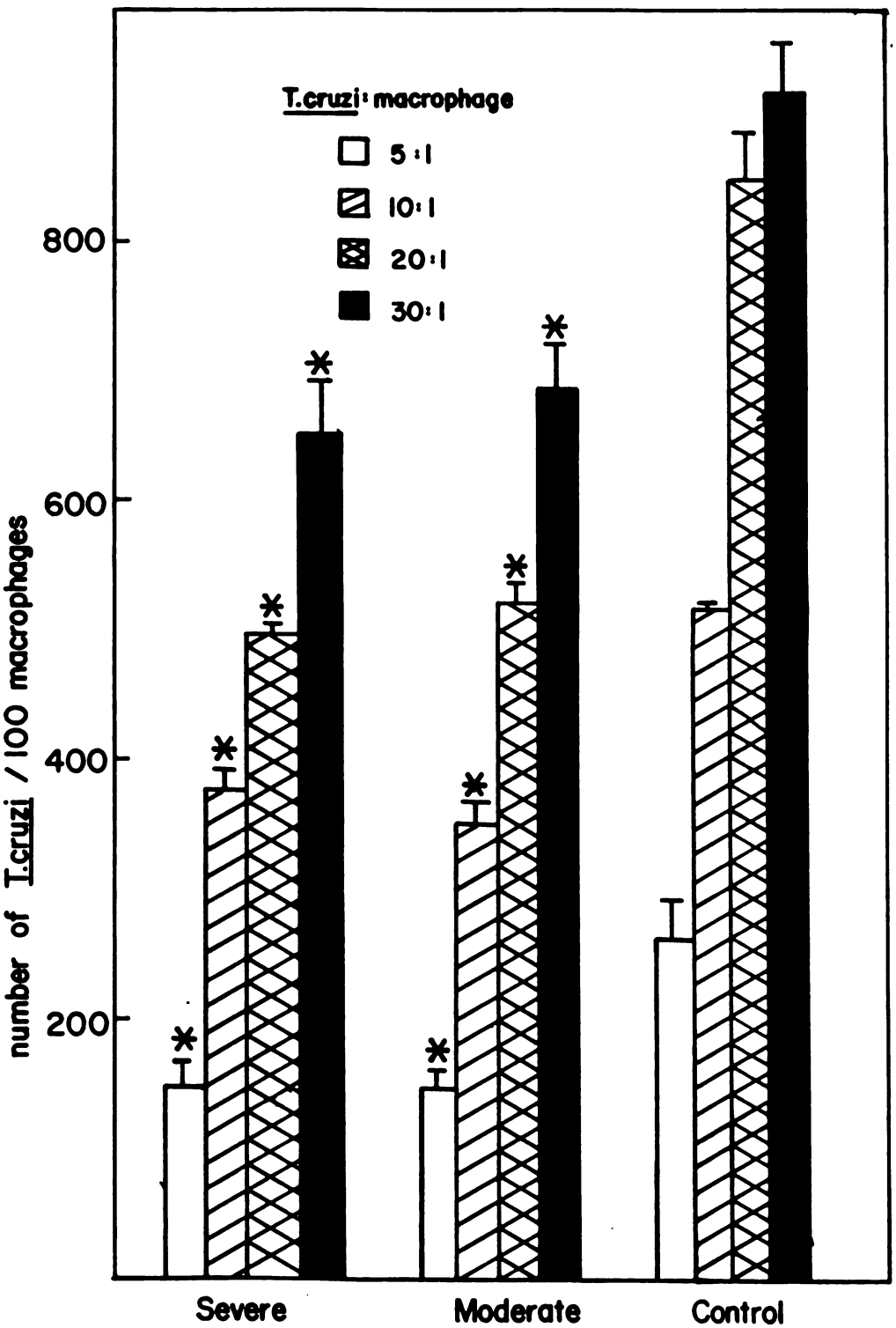

T.cruzi : macrophage
5 : 1
10 : 1
20 : 1
30 : 1
nmoles H_2O_2/mg macrophage protein
30
20
10
Severe
Moderate
Control

Figure 6. Number of T. cruzi associated with resident peritoneal macrophages prepared from severely zinc deficient, moderately zinc deficient, or control mice. Macrophages and parasites are the same as those used in figure 6. Trypomastigotes were incubated for 2 hours with macrophages from each dietary group at a T. cruzi:-macrophage ratio of 5:1, 10:1, 20:1, or 30:1 in DMEM. Nonassociated parasites were removed by washing. The remaining parasites and macrophages were stained with Giemsa. The number of parasites and number of macrophages with parasites were counted. Each bar represents mean ± SEM of triplicates of pooled peritoneal cells from 10 to 15 mice. Asterisk indicates significance of $p < 0.05$ or better as compared to the control group for the same T. cruzi:macrophage ratio.


T.cruzi : macrophage
5 : 1
10 : 1
20 : 1
30 : 1
800
600
400
200
number of T.cruzi / 100 macrophages
Severe
Moderate
Control

parasites associated with macrophages from zinc deficient mice is consistent with results in several previous studies (8, 9). The percentage of macrophages from zinc deficient versus zinc adequate mice, which had one or more T. cruzi associated with it, however, was not significantly different (Figure 7). In contrast, previous studies have shown that, at lower levels of association, the percentage of zinc deficient macrophages associated with T. cruzi was also significantly lower than that with zinc adequate macrophages (8, 9). This inconsistency is most likely due to much higher levels of parasites that had to be used in these studies in order to detect H_2O_2 in a quantitative manner. At the high levels of association required for production of good quantities of H_2O_2, the macrophages were always viable for the few hours during the assay for H_2O_2. Furthermore, when the amount of H_2O_2 produced was calculated per number of parasites associated with 100 macrophages in these particular experiments (Figure 8 derived from Figures 5 & 6), there appeared to be no difference in the amount of H_2O_2 produced amoung macrophages from the various dietary groups. For all groups, there was about 3 $\pm$ 1 nmoles H_2O_2/ mg macrophage protein/ T. cruzi associated with macrophages produced. This suggested that deficient macrophages can produce normal amounts of H_2O_2 upon stimulation with T. cruzi but there was simply less stimulant and therefore less peroxide production due to the decrease in number of T. cruzi associated with the deficient macrophages.

The data suggested that the major defect in H_2O_2 production by macrophages from zinc deficient mice was a defect in ability of T.

Figure 7. Proportion of macrophages from severely zinc deficient, moderately zinc deficient, or control mice that were associated with T. cruzi. Macrophages and parasites are the same as those used in figure 6. Trypomastigotes were incubated for 2 hours with macrophages from each dietary group at a T. cruzi: macrophage ratio of 5:1, 10:1, 20:1, or 30:1 in DMEM. Nonassociated parasites were removed by washing. The remaining parasites and macrophages were stained with Giemsa. The number of macrophages with parasites was counted. Each bar represents the mean ± SEM of triplicates of pooled peritoneal cells from 10 to 15 mice.

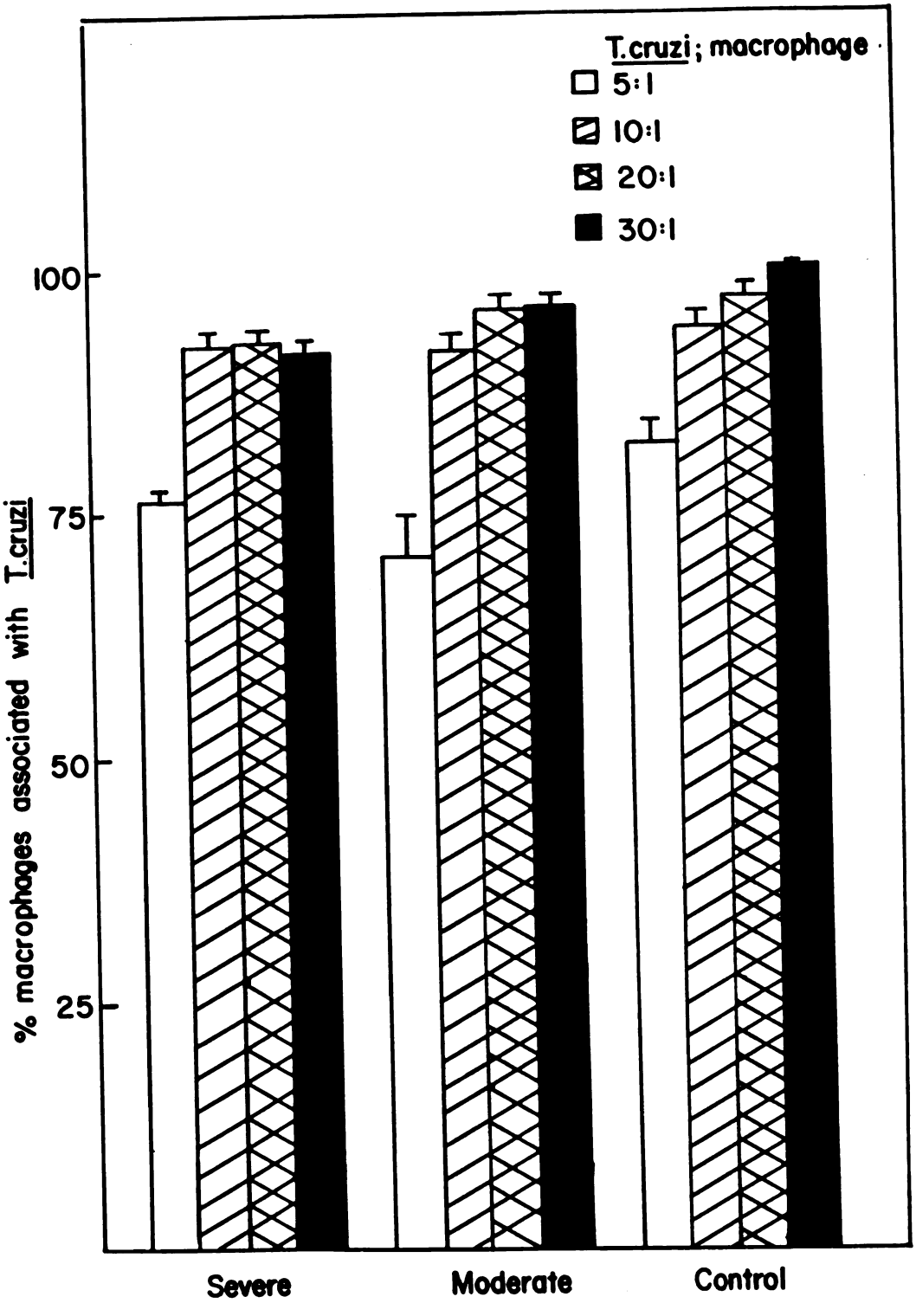
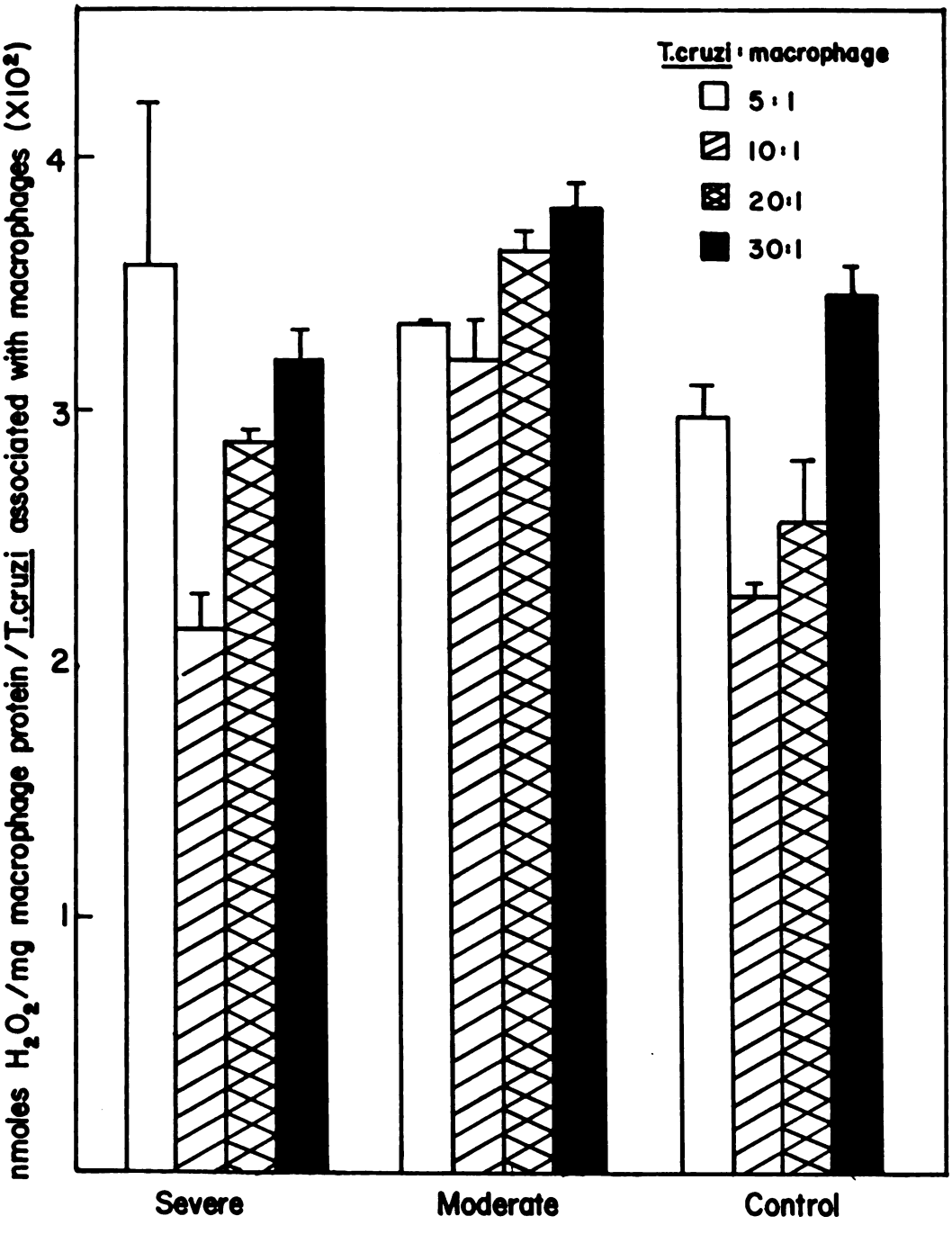

T.cruzi; macrophage
5:1
10:1
20:1
30:1
100
75
50
25
% macrophages associated with T.cruzi
Severe
Moderate
Control

Figure 8. Amount of H_2O_2 produced per T. cruzi associated with resident peritoneal macrophages prepared from severely zinc deficient, moderately zinc deficient, or control mice as calculated from figures 6 and 7. (nanomoles H_2O_2/mg macrophage protein)/(number of T.cruzi/100 macrophages).


T.cruzi : macrophage
5 : 1
10 : 1
20 : 1
30 : 1
nmoles H_2O_2/mg macrophage protein / T.cruzi associated with macrophages (X10²)
1
2
3
4
Severe
Moderate
Control

cruzi to associate with the macrophages. The mechanisms for T. cruzi penetration of macrophages and phagocytosis of T. cruzi by macrophages is unknown. In an attempt to explain the deficit in association of deficient macrophages with T. cruzi, we analyzed fatty acid composition of phospholipids from zinc deficient macrophages and T. cruzi stimulated 20:4 release by zinc deficient macrophages. The relative proportions of the fatty acids in the macrophage phospholipids were the same in the zinc deficient, restricted, and zinc adequate groups (Figure 9). The relative ratios for 16:0, 18:0, 18:1, 18:2 to 20:4 were 4:3:1:1:3. Thus about 25% of the fatty acid of the phospholipids was 20:4. The total amount of each fatty acid in the phospholipids was also the same for macrophages from zinc deficient and zinc adequate mice (Figure 10). In conclusion, the reduced association of T. cruzi to macrophages from zinc deficient mice was not due to an altered composition of fatty acids in the macrophage phospholipids.

Although the fatty acid composition of the macrophage phospholipid was normal, the possibility remained that T. cruzi-stimulated release of fatty acid metabolites was reduced in macrophages from zinc deficient mice. To analyze the release of 20:4 metabolites, adherent resident macrophages from mice in each dietary group were incubated overnight with ^{3}H-20:4. Then, T. cruzi-stimulated release and nonstimulated spontaneous release of 20:4 and its metabolites into the medium were analyzed. Since the fatty acid composition of the macrophage phospholipids was normal for zinc deficient mice, it was likely that deficient macrophages would incorporate ^{3}H-20:4 in

Figure 9. Mole percent of fatty acids in phospholipids from resident peritoneal macrophages prepared from severely zinc deficient, moderately zinc deficient, restricted, or control mice. Adherent macrophages were scraped into 0.15 M NaCl. Margaric phosphotidlycholine was added as an internal standard. Lipids were extracted by the Bligh and Dyer method. Phospholipids were separated from neutral lipids using a silicic acid column. Then, the fatty acids were transmethylated and quantitated by gas liquid chromatography techniques. Protein content of the scraped cells was determined by the Lowry method. Each bar represents the mean $\pm$ SEM of triplicates of pooled peritoneal cells from 10 to 15 mice.

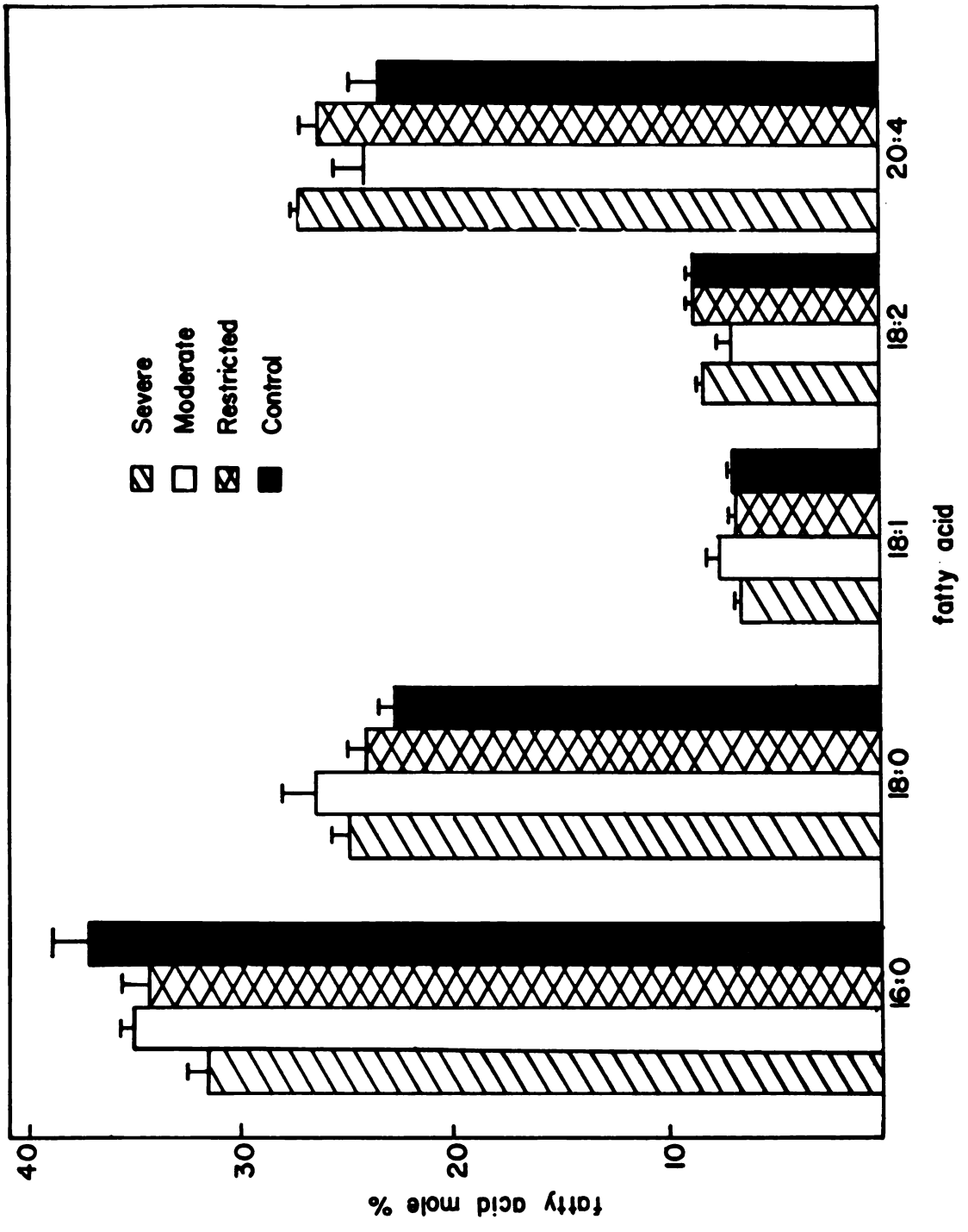
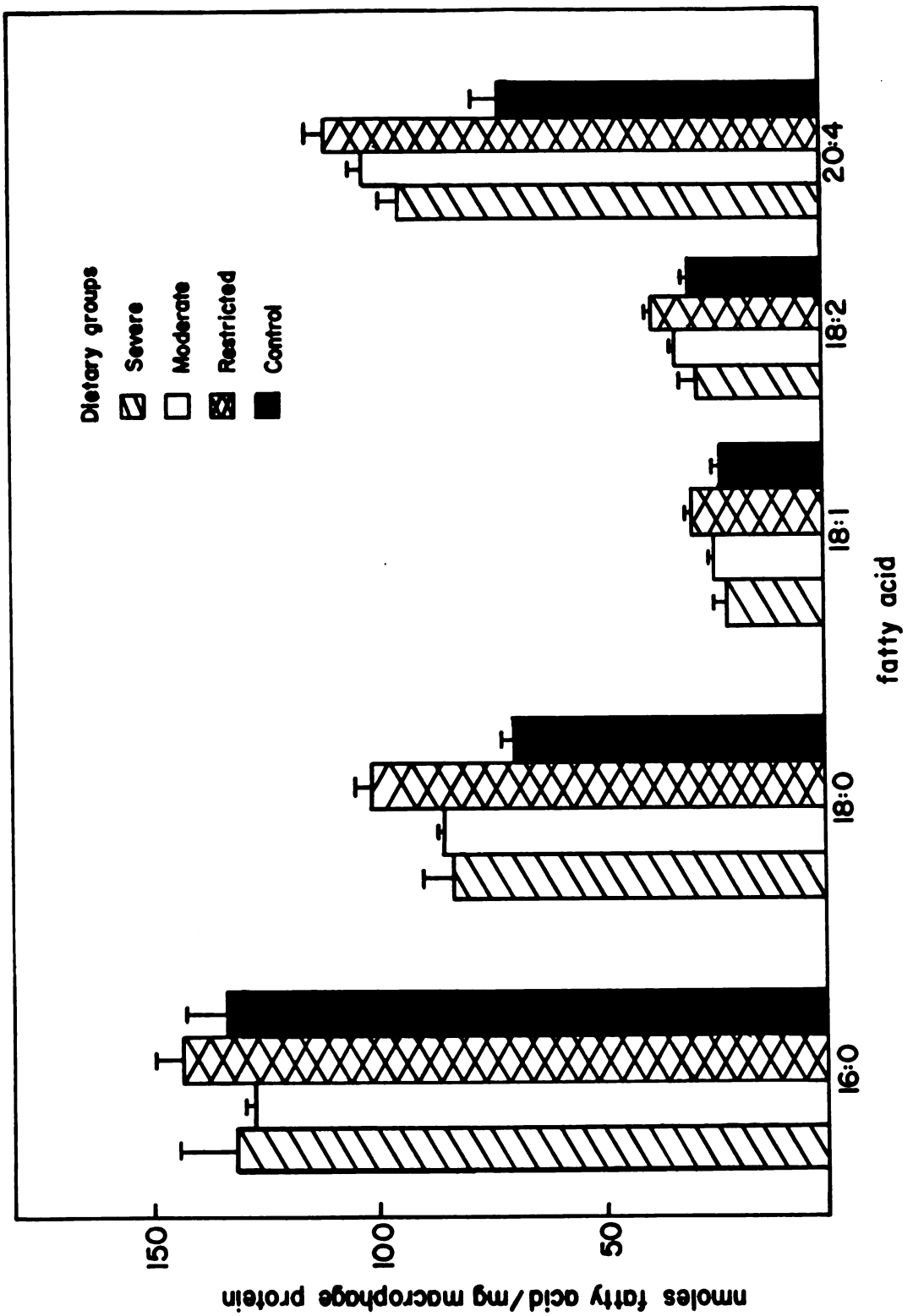

Severe
Moderate
Restricted
Control
40
30
20
10
fatty acid mole %
16:0
18:0
18:1
18:2
20:4
fatty acid

Figure 10. Nanomoles fatty acid per milligram macrophage protein in phospholipids from resident peritoneal macrophages prepared from severely zinc deficient, moderately zinc deficient, restricted, or control mice. The nanomoles of each fatty acid was calculated for figure 10 using the peak area of the internal standard. Each bar represents the mean $\pm$ SEM of triplicates of pooled peritoneal cells from 10 to 15 mice.


nmoles fatty acid/mg macrophage protein
150
100
50
16:0
18:0
18:1
18:2
20:4
fatty acid
Dietary groups
Severe
Moderate
Restricted
Control

the same manner as the controls. Table 1 shows that indeed the zinc deficient macrophages incorporated ^{3}H-arachidonic acid into the same phospholipids and to the same extent as control macrophages. However, T. cruzi stimulated release of 20:4 and it's metabolites from zinc deficient macrophages was altered (Table 2). Although the same amount of PGE_2 and HETE's were released by macrophages from zinc deficient and zinc adequate mice (Table 2), there was a 319% and 249% increase in radioactivity for the severely and moderately deficient groups as compared to the zinc adequate control at a Rf of 0.02 on the thin layer chromatogram (Table 2). This peak of radioactivity may be leukotrienes since leukotrienes were comigratory. However, the total release of ^{3}H was small (about 2%) compared to the amount incorporated into the macrophage phospholipids.

TABLE 1

Incorporation of ^{3}H-arachidonate into Phospholipids[a]

Dietary Group	^{3}H (cpm)[b]
Severely Zinc Deficient	44680 ± 4727
Moderately Zinc Deficient	50934 ± 8191
Restricted	34972 ± 2763
Zinc Adequate	49256 ± 9912

[a] Adherent macrophages from 2 x 10^{6} peritoneal cells prepared from severely zinc deficient, moderately zinc deficient, restricted, or zinc adequate mice were incubated with 1 μCi of (5, 6, 8, 9, 11, 12, 14, 15-^{3}H) arachidonic acid overnight. Excess ^{3}H-arachidonic acid was removed by washing. Macrophages were collected by scraping, and the lipids extracted. Lipids were separated by thin layer chromatography in a solvent system of chloroform:methanol:acetic acid:water (25:20:3:0.3, v:v:v:v). Plates were scraped in 0.5 cm ascending segments for each sample and ^{3}H measured by scintillation counting. The amount of macrophage protein per sample was the same for the severely zinc deficient, moderately zinc deficient, restricted, and zinc adequate groups.

[b] Mean ± SEM of the radioactivity in the one radioactive peak at Rf = 0.94 for triplicates of pooled peritoneal cells from 12 to 20 mice from each dietary group.

TABLE 2

Release of ^{3}H-arachidonate metabolites[a]

Rf[b]	Comigratory Standards	^{3}H (cpm)			
		Severe[c]	Moderate	Restricted	Control
0.02	LTC_4, LTD_4, LTE_4	964 ± 16[d,e]	751 ± 22[e]	345 ± 60	302 ± 48
0.35	PGE_2	179 ± 13	153 ± 24	142 ± 14	150 ± 14
0.67	HETE's	166 ± 54	121 ± 20	123 ± 5	84 ± 48

[a] Release of ^{3}H-arachidonate metabolites form adherent resident macrophages stimulated with T. cruzi at a T. cruzi:macrophages ratio of 25:1. Briefly, medium removed from the adherent macrophage monolayer was centrifuged. Supernatants were acidified with 0.03 M citric acid and extracted three times with chloroform: methanol (2:1, v:v). The chloroform layer was washed with methanol:water (2:1, v:v), evaporated to dryness under N_2 and applied to silicic acid coated plates. The plates were developed twice in chloroform:ethylacetate:methanol:acetic acid:water (140:60:16:2:1, V:V:V:V:V). Plates were scraped in 0.5 cm ascending segments for each sample and ^{3}H measured by scintillation counting.

[b] Rf of standards: 0.02 = (LTC_4, LTD_2, LTE_4), 0.28 = TxB_2, 0.32 = 6-keto-PGF_2, 0.35 = PGE_2, 0.52 = LTB_4, 0.67 = HETE's, 0.79 = ricinoleic acid, 0.88 = arachidonate

[c] Dietary groups

[d] Mean of (cpm of ^{3}H of peak - cpm of spontaneous release) ± SEM for triplicates of pooled peritoneal cells from 12 to 20 mice from each dietary group. Spontaneous release of 86 ± 27 cpm or leukotrienes, 74 ± 14 cpm for PGE_2, and 160 ± 32 cpm for HETE's was the same for macrophages from mice in all dietary groups.

[e] Significance of $p < 0.05$ or better as compared to control group

DISCUSSION

Since zinc deficient macrophages are unable to destroy the T. cruzi associated with them (7, 8) and H_2O_2 production is deemed important in the destruction of T. cruzi, it was hypothesized that macrophages from zinc deficient mice produced less H_2O_2 per amount of stimulant than macrophages from zinc adequate controls. However, it was shown that macrophages from deficient mice produced normal amounts of H_2O_2. Upon stimulation with the commonly used agent, PMA, zinc deficient macrophages produced as much H_2O_2 as zinc adequate macrophages. We also exogeneously added a more natural agent, opsonized zymosan, or the second messenger in opsonized zymosan stimulation of NADPH oxidase, 20:4, to stimulate H_2O_2 production. Again, zinc deficient macrophages produced normal amounts of H_2O_2 as compared to zinc adequate macrophages when stimulated with opsonized zymosan or 20:4. Since PMA and opsonized zymosan stimulate NADPH oxidase through different mechanisms (46-58), it was possible that T. cruzi stimulates NADPH oxidase through yet another mechanism. Therefore, it was important to use T. cruzi as the stimulant for H_2O_2 production. It was shown that macrophages from severely and moderately zinc deficient mice produced significantly less H_2O_2 per macrophage (63% to 73% of control) than zinc adequate macrophages. However, this was determined to be due to less overall activation of the deficient macrophages since fewer parasites had associated with these cells. When considered from this point of view, the amount of H_2O_2 produced per parasite was the same for macrophages from each dietary group of

mice. This relationship between association and H_2O_2 production is in agreement with the results in chapter 4 where the amount of H_2O_2 produced correlated with the level of T. cruzi-macrophage association. Furthermore, one would expect that destruction of a lower burden of parasites such as that associated with the deficient macrophages would require less H_2O_2. With this assumption, the conclusion can be made that the mechanism for production of H_2O_2 is unaltered in the macrophages from zinc deficient mice. The macrophages had probably not undergone repair in vitro since, in all studies herein, the amount of zinc in the cultures was less than 1.5 μg Zn/ dl and similar amounts were present in previous studies where microbicidal activity of deficient macrophages was aberrant (7, 8). As a comparison, 1000 μg $ZnCl_2$/dl which is about 500 μg zinc/dl was used in other studies to restore microbicidal activity by macrophages from zinc deficient mice to control levels (7, 8). Therefore, the absense of dietary zinc must alter some other microbicidal process of the macrophages since previous studies showed that macrophages from zinc deficient mice had a reduced ability to destroy those parasites that had associated with it (7, 8).

Unfortunately, the amount of killing of the parasite by the macrophages from deficient and control mice could not be measured since in the first experiment the level of T. cruzi-macrophages association was too low for accurate measurement. When repeated at the higher level of infection required for stimulation of good levels of H_2O_2 production, the macrophages were destroyed by the parasites after the 6 to 18 hours, when degree of killing of T. cruzi was

measured. As mentioned before, the degree of infectivity of the parasite is highly variable and unpredictable. However, herein, the macrophages were prepared under the same zinc free conditions as in previous studies where it was shown that there was a reduction in association of T. cruzi with and killing of T. cruzi by the macrophages from zinc deficient mice. Also in this study, the association of T. cruzi with the zinc deficient macrophages was reduced. So, one may assume that in this study, the microbicidal activity of the macrophages from zinc deficient mice was also aberrant.

A high percentage of macrophages (85-100%) associated with parasites were used when it was shown that H_2O_2 production per parasite was normal for deficient macrophages. In contrast, in previous studies, lower levels of association (18-45%) were used to show that killing of T. cruzi by zinc deficient macrophages was reduced compared to controls. Thus, it is important to show that, also, at lower levels of association, H_2O_2 production per parasite was the same for the deficient and control groups. The two studies herein, where the T. cruzi-macrophage association was very low (10 $\pm$ 5% of the macrophages were associated with parasites), H_2O_2 production by macrophages from zinc deficient mice was 63% to 73% of controls and, in previous studies, association with T. cruzi by deficient macrophages was in the range of 50% to 75% of controls. Therefore, even at low levels of association, the ratio between deficient and control groups for H_2O_2 production and for association was the same. Thus, the amount of H_2O_2 per parasite was probably the same among the dietary groups at both low and high levels of infection.

Still, previous evidence (9-11) and the data in Chapter 4 suggest that H_2O_2 is important in destruction of T. cruzi. Perhaps some process in the killing following the production of H_2O_2 requires zinc. However, the actual process of destruction of T. cruzi and whether or not some agent in addition to H_2O_2 is important remains to be determined.

The reduced association of parasites with macrophages from zinc deficient mice compared to controls was also hypothesized to be due to a reduction in long-chain unsaturated fatty acids such as 20:4 since this has been shown to reduce membrane fluidity and phagocytosis (23, 24). If fatty acid composition is altered in deficient macrophages and it is important in T. cruzi-macrophage association, the macrophages from zinc deficient mice must also be able to restore the fatty acid composition to normal in a half hour incubation with zinc followed by a one hour incubation with T. cruzi since, in this time span with zinc, the association of T. cruzi with macrophages from zinc deficient mice is restored to control levels (7, 8). Synthesis and incorporation of new fatty acids requires too much time (59). However, the entire macrophage membrane is recycled via pinocytosis every 33 minutes (60). Perhaps, during the recycling of the membrane which has been suggested to involve the Golgi apparatus and the endoplasmic reticulum (61-63), the existing fatty acids can be modified to restore the fatty acid composition to normal. However, the hypothesis that fatty acids were altered was incorrect. The fatty acid composition and total amount of each of the fatty acids of the phospholipids from zinc deficient macrophages was the same as

controls. The presence of normal amounts of 20:4 in phospholipids of deficient macrophages is in agreement with the finding that opsonized zymosan stimulated production of normal amounts of H_2O_2 by zinc deficient macrophages since 20:4 is a second messenger in opsonized zymosan stimulation of H_2O_2 production (48, 49, 54, , 46, 47).

Although the membrane fatty acid composition is normal for zinc deficient macrophages, the T. cruzi stimulated release of 20:4 metabolites, which may be involved in association and killing of T. cruzi (7, 8), may by altered. Zinc deficient macrophages and zinc adequate macrophages released normal amounts of PGE_2 and HETE's, ^3H-arachidonate metabolites, into the culture medium upon stimulation with T. cruzi. Spontaneous release of PGE_2 and HETE's was very low for each dietary group. However, these preliminary results did indicate that there was an increase in what may be leukotrienes. This was not due to altered incorporation of ^3H-arachidonic acid since the same amount of radioactivity was incorporated into phospholipids of mononuclear cells from zinc deficient and zinc adequate mice. Also, the release of this 20:4 metabolite was several times the spontaneous release by nonstimulated macrophages from all dietary groups. Since this radioactive peak comigratory with leukotrienes was very close to the origin, it could be phospholipids instead of leukotrienes. However, one would not expect viable macrophages to release large amounts of entire phospholipids upon stimulation with T. cruzi. This data is very preliminary and identification of the increased production of this 20:4 metabolite that was comigratory with leukotrienes must be verified by high pressure liquid

chromatography. Also, the total release of ^3H was small (about 2%) compared to that incorporated into the macrophage phospholipids. Thus, T. cruzi may be less stimulatory than other agents such as zymosan reported to cause release of 6% to 15% of the tritium labelled 20:4 metabolites from macrophage phospholipids. Another possibility is that optimal conditions for T. cruzi stimulation of macrophages were not used. Further, one would expect leukotriene production to be reduced in deficient macrophages since exogenous addition of LTC_4 or LTB_4 have been shown to increase not decrease T. cruzi-macrophage association (25, 26), killing of T. cruzi by macrophages (25, 26), and stimulation of the "oxygen burst" (54, 64). Although from the data collected to date, it is also not known if the supposed leukotrienes were active. Leukotrienes can be oxidized by the oxygen metabolites of the burst (65). In the interim, no sound conclusions can be made about leukotrienes until further studies are done to identify the 20:4 metabolites produced.

In summary, zinc deficient macrophages produced normal amounts of H_2O_2 per amount of stimulant. The reduced association of T. cruzi with macrophages from zinc deficient versus zinc adequate mice was not the consequence of altered fatty acid composition of the deficient macrophages. Also, preliminary data suggests that T. cruzi-stimulated release of an 20:4 metabolite was three times greater by macrophages from zinc deficient mice compared to controls. The potential importance and role of this metabolite in association and destruction of T. cruzi by macrophages should be the subject of future studies.

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SUMMARY AND CONCLUSIONS

Zinc deficiency, not infrequently encountered in humans, drastically compromizes cell and antibody mediated immune responses in both humans and animals. The reduced immune capacity is at least in part due to a 50% decrease in number of leukocytes in animals. The functional capacity of the residual cells may also be altered upon suboptimal intake of zinc since over 100 enzymes are dependent upon zinc for activity. Therefore, the purpose of this research was to examine the functional capacity of the residual leukocytes in an attempt to identify an aberrant zinc dependent process. In all these studies, the possibility for in vitro repair due to addition of serum zinc was minimized by using autologous serum from zinc deficient mice (50 μg Zn/dl) or a serum free system rather than fetal calf serum which contains relatively high levels of zinc (350 μg Zn/dl) and is commonly used for culturing leukocytes.

The results of these experiments showed that residual splenic lymphocytes from zinc deficient mice could function at least as well as splenocytes from zinc adequate mice. Proliferation and production of interleukin 2 (IL-2) in repsonse to the T-cell mitogen concanavalin A was the same for splenocytes from severely and moderately zinc deficient mice as for splenocytes from zinc adequate and resticted mice. These results are in agreement with a previous report on Con A proliferation by splenocytes from zinc deficient mice where the in vitro amount of zinc was limited. However, other reports showing

reduced proliferation in response to Con A for the deficient group contradict the results herein. This is probably due to their depleting zinc in vitro instead of dietary depletion of zinc. With in vitro depletion, there is simply removal of zinc from the existing cells whereas depletion of zinc in vivo may also affect cell development as well as cause production of factors such as cortisone from other tissues which may play a part in the deficiency. During a T-cell response to allogeneic cells in a one-way mixed lymphocyte culture (MLC), splenocyte proliferation, IL-2 production by splenocytes, and the number of splenocytes with IL-2 receptors for the zinc deficient groups was twice that of the zinc adequate groups. This increased response to allogeneic cells may be due to alterations in the proportions of T-cell subsets since relatively immature T-cells respond in a primary MLC whereas concanavalin A, a polyclonal activator, stimulates many T-cell subsets. This possibility could be analyzed in the future by fluorescently labelling cell surface markers. Another possible explanation for the increased response in the MLC may be that some inhibitory activity is impaired in splenocytes from zinc deficient mice.

Residual B-cells from zinc deficient mice also seemed to function normally. Splenocytes of zinc deficient mice activated in vivo with sheep red blood cells produced the same amount of IgM and IgG per IgM and IgG plaque forming cell (PFC, antibody secreting cell), respectively, as splenocytes from zinc adequate mice. Also, in agreement with many previous reports, the number of PFC per million splenocytes was the same among the experimental dietary groups

although the number of PFC per spleen was reduced (about 50% of control) for the deficient groups. In conclusion, residual splenic lymphocytes from zinc deficient mice retained normal functional capacity at least for those responses tested.

In contrast, resident peritoneal macrophages from zinc deficient mice have a reduced capacity to associate with and destroy the obligate intracellular parasite T. cruzi which causes Chagas' disease in humans. Since H_2O_2 is thought to play a critical role in the destruction of T. cruzi, it was important to compare H_2O_2 production by resident peritoneal macrophages from zinc deficient mice with H_2O_2 production by macrophages from zinc adequate mice. However, in order to measure H_2O_2 production by resident macrophages instead of the commonly used in vivo activated macrophages, the assay for measuring H_2O_2 production needed to be improved. Previously, the amount of H_2O_2 produced by resident macrophages was reported to be low to negligable. Also, although a considerable number of studies have been done to link H_2O_2 production to destruction of T. cruzi, T. cruzi-stimulated H_2O_2 has not been directly quantitated.

Modifications that were made in the conditions for the commonly used phenol red assay to optimize H_2O_2 production by resident macrophages stimulated with phorbol or opsonized zymosan included an increase in cell concentration, addition of calcium and an incubation in ambient air at 37°C. However, when the stimulant was the living pathogen T. cruzi, H_2O_2 production could not be measured since the high concentrations of phenol red required for the assay were toxic to T. cruzi. The assay was further modified by using

another substrate homovanillic acid (HVA). To improve the HVA assay for use with resident macrophages, the concentration of macrophages was again increased. Also, $CaCl_2$ and $MgCl_2$, which were included in the previously described assay with HVA, were determined to be required for optimal H_2O_2 production by resident peritoneal macrophages stimulated with phorbol or opsonized zymosan. Using this modified assay, the amount of H_2O_2 produced by resident macrophages could now be quantitated. In addition, with the HVA assay, it was now possible for the first time to quantitate H_2O_2 production by macrophages stimulated with T. cruzi. This assay will be essential for future studies on T. cruzi destruction and may be important when other living pathogens such as Leshmania or Plasmodium falciparum are used to activate H_2O_2 production by macrophages.

T. cruzi-stimulated H_2O_2 production by macrophages from mice consuming normal laboratory chow was studied since there is no available literature on the quantitation or mechanism of T. cruzi-stimulated H_2O_2 production. Resident peritoneal macrophages incubated with amastigotes, the intracellular form of T. cruzi, produced half as much H_2O_2 as macrophages incubated with trypomastigotes, the blood form of T. cruzi. Trypomastigotes that were opsonized with heat inactivated serum from chronically infected mice stimulated four times as much H_2O_2 production as nonopsonized trypomastigotes. Also, upon stimulation with trypomastigotes or opsonized trypomastigotes but not amastigotes, the amount of H_2O_2 produced correlated with the proportion of macrophages associated with parasites and with the number of parasites per macrophage. The maximum amount of

H_2O_2 produced by the macrophages stimulated with trypomastigotes varied. Perhaps the maximal amount of H_2O_2 produced by macrophages depends upon the "stage" of the parasite in its progression through each of the forms of its lifecycle.

Furthermore, a pathway by which T. cruzi trypomastigotes may stimulate H_2O_2 production was analyzed. Preliminary data indicated that T. cruzi trypomastigotes stimulated the release of the arachidonate (20:4) metabolites PGE_2, HETE's and what may be leukotrienes in a ratio of 2:1:4. HETE's and leukotrienes are known to stimulate the production of oxygen metabolites such as H_2O_2. Therefore, 20:4 and its metabolites may be second messengers in the production of H_2O_2 by resident macrophages. In addition, as a source for production of 20:4 metabolites, resident peritoneal macrophages from A/J mice contained a considerable amount of endogeneous 20:4 (21% of the fatty acid) in the phospholipids. If trypomastigotes also stimulate the release of shorter-chain, more-saturated fatty acids than 20:4, these fatty acids would not activate nearly as much H_2O_2 production as 20:4 and perhaps its metabolites since 20:4 stimulated 2 to 3 times as much H_2O_2 production as 18:0, 18:2, or 18:3. Taken together, the data suggested that 20:4 and its metabolites may be intermediates in one pathway for trypomastigote-stimulation of H_2O_2 production by resident macrophages.

Now that the amount of H_2O_2 produced by T. cruzi-stimulated macrophages could be measured and preliminary data indicated one possible mechanism for T. cruzi-stimulation of H_2O_2 production, possible alterations in the ability of resident macrophages from

dietary zinc deficient mice to produce H_2O_2 could be studied. It was shown that, upon incubation with T. cruzi trypomastigotes, the total amount of H_2O_2 produced per macrophage from severely and moderately zinc deficient mice was 66% and 83%, respectively, of that by macrophages from zinc adequate mice. However, this reduction in H_2O_2 production was due to less stimulation of the macrophages from zinc deficient mice since fewer T. cruzi associated with the deficient macrophages. Thus, the amount of H_2O_2 produced per T. cruzi associated with the macrophages was the same for all experimental dietary groups. One would expect that a reduced number of parasites would also require proportionally less H_2O_2 for destruction. The H_2O_2 production by macrophages stimulated with the commonly used nonliving agents phorbol, opsonized zymosan, or 20:4 was also the same for all dietary groups. Therefore, at a given amount of stimulant, H_2O_2 production by macrophages from A/J mice was not altered by deprivation of dietary zinc. Since, macrophages from zinc deficient mice destroyed a smaller proportion of the parasites associated with them than macrophages from zinc adequate or restricted mice, some process in the destruction of T. cruzi besides the production of H_2O_2 must directly or indirectly require zinc. Perhaps zinc is required in some process in killing following production of H_2O_2.

The mechanisms for T. cruzi invasion of macrophages and macrophage phagocytosis of T. cruzi is unknown. However, since a decrease in long chain unsaturated fatty acids is known to decrease phagocytosis and since exogeneous addition of leukotrienes, 20:4 metabolites, are known to increase the association of trypomastigotes

with macrophages, it was hypothesized that the reduction in number of trypomastigotes associated with deficient macrophages may be due to an altered fatty acid composition. It was shown that the ratios among and total amount of 16:0, 18:0, 18:1, 18:2, and 20:4 were unaltered in the phospholipids of macrophages from zinc deficient mice as compared to macrophages from zinc adequate or restricted mice. Thus, the reduced association of T. cruzi with macrophages from zinc deficient mice was not due to an altered composition of fatty acids in the phospholipids. Preliminary data suggested that the amount of release of the 20:4 metabolites, HETE's and PGE_2, was the same for all experimental dietary groups. However, macrophages from zinc deficient mice released three times as much of an 20:4 metabolite, that may be some leukotriene, than macrophages from zinc adequate mice. Identification of this metabolite and its role in the association of T. cruzi with macrophages requires futher study.

In summary, dietary zinc deprivation did not reduce several functions of residual lymphocytes from zinc deficient mice but did reduce some functions of residual peritoneal macrophages from zinc deficient mice. The reduced ability of deficient macrophages to destroy T. cruzi was not the result of reduced production of H_2O_2 per given amount of stimulant. Also, the reduced T. cruzi-macrophage association for the experimental deficient group was not a consequence of an altered fatty acid composition within the macrophage phospholipids or the result of reduced production of leukotrienes. In fact, compared to controls, macrophages from zinc deficient mice produced three times as much of an 20:4 metabolite. In

conclusion, some other processes must be aberrant in the association with and destruction of T. cruzi by macrophages from zinc deficient mice. Also, an important contribution was made to the study of T. cruzi destruction by leukocytes since the improved assay conditions for H_2O_2 production now makes it possible to directly quantitate the amount of H_2O_2 produced by leukocytes stimulated with T. cruzi.

APPENDIX

Composition of the Zinc Deficient and Zinc Adequate Diet

The zinc deficient diet (Table 1) was designed and extensively examined by Luecke, R. W. and Fraker, P. J. ((1979)J. Nutr. 109, 1373-1376). Briefly, it was shown that A/J female mice fed zinc deficient diet supplemented with 5.9 to 31.4 μg Zn/g attained maximum growth and had normal antibody-mediated responses to sheep red blood cells. However, drastic reductions were observed for the antibody-mediated responses by splenocytes from A/J mice fed the zinc deficient diet containing less than 1 μg Zn/g. Therefore, in the studies described in this thesis, zinc deficient diet contained less than 1 μg Zn/g and the zinc adequate diet contained 27 μg Zn/g.

Table 1

Composition of the zinc deficient diet

	g/kg
Glucose monohydrate	590
Egg white solids (spray-dried)[1]	220
Corn oil	100
Fiber[1]	30
Salt mix[2]	40
Vitamin mix[3]	10
Ethoxyquin[4]	10

[1]Cellulose-type fiber, Teklad Test Diets, Madison, Wisconsin, [2]Bernhart, F. W. & Tommarelli, R. M. (1966) J. Nutr. 89, 495, except that a U.S.P. grade of $CaHPO_4$ was used instead of reagent grade, and also zinc carbonate was omitted. [3]Composition similar to the AIN-76 mixture (1977) J. Nutr. 107, 1340, except that the biotin level was increased to provide an additional 4 mg/kg diet. [4]Santoquin, Monsanto Chemical Co., St. Louis, Missouri.