MODULATION OF MACROPHAGE-*TRYPANOSOMA CRUZI* INTERACTION BY LACTOFERRIN

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ABSTRACT

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Chagas' disease is a chronic debilitating illness caused by the flagellated protozoan Trypanosoma cruzi. This disease currently affects 20 million people in Latin American countries. In its acute phase Chagas' disease presents intense inflammatory cell infiltrates around ruptured parasitized cells. Lactoferrin, an iron-binding protein, is secreted by neutrophils (cells present in acute chagasic lesions) upon stimulation. Lactoferrin levels increase during inflammatory conditions. In this work, evidence is presented that lactoferrin modulates the outcome of the interaction between mouse peritoneal macrophages or human monocytes and the intracellular amastigote or bloodstream trypomastigote forms of T. cruzi, increasing the ability of the phagocytic cells to bind and kill the parasites. The latter capacity was found to be dependent on the presence of iron in the protein. Pretreatment of human monocytes, mouse peritoneal macrophages and amastigotes with lactoferrin also increased their mutual association. These phagocytic cells and amastigotes present receptors for this protein. Pretreatment of macrophages with lactoferrin and subsequent exposure to agents that bind to the protein, prior to exposure to parasites, did not abrogate the lactoferrin effects, whereas complete inhibition of the effect was

seen when the blocking agents were present in the medium together with lactoferrin and cells. The lactoferrin effect was also abrogated when the parasites were pretreated with lactoferrin, exposed to blocking agents and then incubated with the phagocytic cells. Macrophages exposed to lactoferrin killed parasites at a faster rate than cells not exposed to the protein. This effect appeared to be due to the production of oxygen metabolites, since parasite killing was inhibited by scavengers of $O_2^{\cdot-}$, H_2O_2 and $^1O_2$. The ability of lactoferrin to stimulate phagocytic function was also observed when invasive trypomastigotes were used instead of amastigotes. Some differences, however, were observed: a) macrophages required a longer lactoferrin treatment (24 hr) for the effect to be seen (as opposed to 1 hr when amastigotes were used); b) the mechanism of killing involved primarily hydrogen peroxide production since only catalase was found to inhibit the effect. The role of lactoferrin iron ions in the ability of this glycoprotein to stimulate macrophage functions was studied next. Apolactoferrin (iron free lactoferrin) or lactoferrin at 20% or 100% iron saturation increased the uptake of amastigotes by macrophages. While the latter two preparations were able to stimulate parasite killing, apolactoferrin has lost this ability. Complete restoration of the effect on killing was afforded by the addition of ferric and ferrous ions and partial restoration with zinc ions. Cupric ions were ineffective. Transferrin, another iron-binding protein, was unable to increase the uptake or killing of the parasite by macrophages. Iron and the $OH^{\cdot}$ radical were involved in the lactoferrin mediated killing effect, since, in the presence of chelators of the former and scavengers of the latter, killing was inhibited. In another series of

experiments, it was found that lactoferrin levels were increased in the sera of mice infected with T. cruzi. The protein was also found bound to amastigotes from the spleen. These results demonstrate that lactoferrin may contribute to the clearance of T. cruzi by phagocytic cells via stimulation of parasite uptake and killing and that lactoferrin function is dependent upon its iron content.

To Fernando and Rachel with love

To my parents with admiration

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PREFACE

Tropical diseases are the great forgotten maladies of our times. No matter that in the third world, where 75% of the world's population lives, they remain one of the major causes of death. This is not the world to which I came to do research nor is it the world that I remember when I think of home. For I too am among the privileged, having had access to adequate shelter, food, and education, which is more than two-thirds of the people living in my country can expect, even today. Given my many privileges, it is fitting that I chose for my studies one of the six diseases singled out by the World Health Organization Special Programme for Research and Training in Tropical Diseases, Chagas' disease.

The work which I carried out in this country, using the many facilities which I now take for granted, is not likely to lead directly to a cure for this disease, nor has it contributed to producing a vaccine, the hope of many. My work, however, is a piece of the puzzle of scientific knowledge necessary to a complete understanding of a complex biological process. I can only hope that this piece falls in the right place.

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ABBREVIATIONS

Apo-LF	Iron-free lactoferrin
α-MM	Alpha methyl mannoside
AMA	Amastigote forms of *T. cruzi*
BSA	Bovine serum albumin
DMEM	Dulbecco's modified Eagle minimum essential medium
DF	Deferoxamine mesylate
DTPA	Diethylene triamine pentaacetic acid
d-FBS	Dialyzed fetal bovine serum
FBS	Fetal bovine serum
HBM	Human blood monocyte
KTBA	α-ketobutyric acid
LF	Human lactoferrin
MEM	Eagle's minimal essential medium
ML-15H	Modified Leibowitz medium containing 2 mg/L of hemin
MPM	Mouse peritoneal macrophages
PBS	Phosphate buffered saline
SOD	Superoxide dismutase

INTRODUCTION

INTRODUCTION

I. Chagas' disease: an overview

Trypanosoma cruzi is the flagellated protozoan that causes Chagas' disease, or American trypanosomiasis (1), a chronic debilitating illness mostly confined to Latin American tropical and subtropical countries. However, some cases have been reported from temperate areas of North America (2,3). Recently, the disease, which was limited to rural areas of the affected countries where the vector, bugs of the family Reduvididae, is normally encountered, has been spreading to more populated areas via transmission by blood transfusion. It has been estimated that up to 20% of blood donors in nonendemic urban areas are infected with T. cruzi (4). About 65 million people are at risk of T. cruzi infection (5). Of these, 15 to 20 million are actually infected. Approximately 10% of the infected individuals, are expected to develop chronic chagasic cardiopathy. In some areas, chronic Chagas' disease may be responsible for up to 10% of the deaths among the adult population (6).

The life cycle of the parasite begins when triatomine bugs deposit feces containing the metacyclic trypomastigote forms of the parasite on the skin of the host. Man acquires the infection by rubbing the contaminated feces into broken skin, mucosa, or the lesion left by the insect bite. The trypomastigotes then gain access to local cells where they transform intracellularly to amastigote forms and multiply by binary fission. After extensive multiplication, some

of the parasites differentiate into trypomastigotes and, upon cell rupture, escape. Released amastigotes, which are thought not to be infective, are disposed of by phagocytic cells. Trypomastigotes, on the other hand, can infect neighboring cells or reach the circulation where they can be ingested by the vector. Once back in the vector, the trypomastigotes migrate to the anterior portion of the digestive tract where they transform into epimastigotes, the dividing form in the insect. In the hindgut, epimastigotes transform into metacyclic trypomastigotes, the infective stage for mammalian hosts.

The acute phase of Chagas' disease encompasses the clinical signs found within the first four months of infection in man and can be diagnosed by the demonstration of parasites in the blood (7). Fever is an early symptom, and is usually accompanied by malaise and headaches (8). Systemic alterations include subcutaneous edema, increase in the volume of the lymph nodes, hepatomegaly, splenomegaly, and often acute myocarditis with characteristic alterations in the electrocardiographical pattern (9). Anemia with low levels of iron, leukocytosis with neutropenia and lymphocytosis are also present (10). Clinical symptoms of Chagas' disease are not always evident. The infection usually takes a more severe course in children and a higher mortality index is found during early infancy (9). In general, mortality during the acute disease is low, around 5 to 10% (9).

The large majority of patients lapse into a phase of asymptomatic infection that can last for years. Some of the subjects present mild anomalies, such as cardiac conduction disturbances and esophageal and colonic disperistalsis (10).

Chronic manifestations of the disease are commonly found many years after the initial infection and include progressive diffuse fibrosing myocarditis that leads to cardiomegaly, cardiac failure, arrythmias, and thromboembolic phenomena (10). Once symptomatic, the course of the disease is grave, with death occurring within a year (10). In some countries, the digestive system of some patients may also be compromised, presenting megaesophagus with dilation and loss of peristaltic movements and megacolon with dilation and obstruction (11).

On a cellular level, the response of the host to T. cruzi is of an inflammatory nature. Vianna (12) reported that the intact, parasitized cells were rarely surrounded by inflammatory infiltrates. However, immediately following the rupture of the parasitized cell, a focal inflammatory infiltrate was observed in the immediate area (12). Mononuclear cells predominate in these infiltrates although polymorphonuclear cells and eosinophils are also present (13). The first cells to appear in inflammatory foci are polymorphonuclear cells which sometimes contain phagocytized parasites, followed by an influx of mononuclear cells, lymphocytes and monocytes (14). The cellular infiltrate is accompanied by intense vascular dilation, congestion, edema and sometimes necrosis. In the chronic phase, the intensity of the infiltrate does not relate to the number of parasites (15). It consists predominantly of lymphocytes and is accompanied by fibrosis (15).

II. Factors influencing parasite-host cell interaction

Host-cell binding and penetration by T. cruzi are vital to assure the initial infection as well as to perpetuate the parasite in the

host. However, the mechanisms by which the parasite obtains an intracellular location remain to be elucidated. Treatment of the host cells with trypsin (16), α-mannosidase (17), α-galactosidase (18), and N-acetyglucosaminidase (19) decreases the ability of the parasite to bind to these cells. These results suggest that a glycoprotein(s) might be relevant in the interaction between host cells and parasites. This point is further supported by the observation that treatment of the host cells with lectins results in inhibition of parasite interiorization (20).

With respect to the parasite, ongoing protein synthesis (21), DNA synthesis (22), glycoprotein synthesis (16) and glycoprotein processing (23) are necessary for host cell infection, since inhibitors of these processes inhibit parasite interiorization into the host cells. The presence of sugar residues on the cell surface of different stages of T. cruzi has been demonstrated by using lectins (29,25,26).

That sugar residues may be playing a role in the parasite-host cell interaction has been shown by the fact that N-acetyl glucosamine pretreatment of the parasite inhibits interiorization of the parasite into bovine endothelial cells (27). Some lectins have been reported to exert the opposite effect (28) For example, concanavalin A, wheat germ agglutinin, and phytohemagglutinin have increased parasite attachment to and uptake by macrophages (20,28). Moreover, treatment of the parasite with exoglycosidases markedly enhances their uptake by phagocytic cells and their ability to infect nonphagocytic cells (17,18,19).

Several glycoproteins have been isolated from the surface of T. cruzi in attempts to pinpoint the specific ligand(s) involved in host cell attachment. A 90 kd surface glycoprotein (29) present on all stages of the parasite and found in sera of chagasic patients (30) has been shown to induce partial protection in mice against challenge with trypomastigotes (31). A complex of glycoproteins with an approximate MW of 85 kd, which is specific for the trypomastigote stage, has been purified by wheat germ agglutinin affinity chromatography (32). Antibodies against this complex have been obtained and the IgG fraction extensively inhibits interiorization of trypomastigotes into LLC-MK2 cells (33). Recently, a gene fragment coding for an antigenic determinant of this protein has been cloned (34). The peptide was shown to consist of three highly conserved 9-amino acid repeats. This type of repetitiveness resembles the surface peptides of Plasmodium falciparum (34). The ability of this peptide to induce protection against challenge by T. cruzi has not been determined.

III. Role of inflammatory cells in Chagas' disease

Studies have been conducted with inflammatory cells both to establish their roles in production of host defense and their ability to interact with T. cruzi. Human neutrophils are able to take up and kill T. cruzi amastigotes in vitro (35). The mechanisms of cytotoxicity of these cells for parasites have been linked to their ability to produce toxic oxygen metabolites. Human neutrophils can also destroy antibody-coated epimastigotes, trypomastigotes or insect-derived metacyclic trypomastigotes extracellularly (35-41) via toxic oxygen metabolites (42).

Human eosinophils are also able to ingest and destroy amastigotes (43) and, in the presence of specific antibodies, can destroy trypomastigotes (39) and insect-derived metacyclic trypomastigotes (41) via ADCC. Eosinophil cationic proteins play a role both in the intracellular and extracellular destruction of T. cruzi in vitro (39,43).

Macrophages play a dual role in Chagas' disease; they can serve both as a host cell harboring the parasite, contributing to parasite dissemination in the body, and as effector cells implicated in the destruction and clearance of parasites from infected tissues. Trypomastigotes can enter macrophages by either phagocytosis (44) or cell membrane penetration (45). After being phagocytized, parasites are found initially within phagosomes, to which lysosomes may fuse (44). After 24 hours, amastigotes are found in the cytoplasm of the macrophage (44), suggesting an escape from the phagosomes via their ability to cross membranes. On the other hand, amastigotes can not penetrate cell membranes and their only means of entry is by phagocytosis. Unlike trypomastigotes, amastigotes have never been seen outside of phagocytic vacuoles (46), and, in the vacuoles, they can be killed by intermediates of oxygen reduction, mainly H_2O_2 (46,47).

Several agents have been found to stimulate the uptake and killing of trypomastigotes by mouse peritoneal macrophages. Human fibronectin, a cell surface protein which is increased in inflammatory states, increases the uptake of T. cruzi by macrophages (48) and fibroblasts (49). T. cruzi possesses receptors for this protein (48), which could possibly act by bridging the cells, thus increasing their association. Exposure of macrophages to lymphokines generated by

antigen-stimulated sensitized spleen cells from mice infected with T. cruzi or BCG (50) results in the destruction of internalized parasites. Trypanocidal activity is also induced in these cells by incubating them with supernatants of lymphocytes stimulated with concanavalin A or bacterial lipopolysaccharide (50). Leukotriene B_4 and leukotriene C_4, products of the metabolism of arachidonic acid, are produced in increased amounts by inflammatory cells, such as polymophonuclear leukocytes and macrophages, after stimulation with phagocytic or chemotactic stimuli (51). These metabolites have been shown to increase the capacity of macrophages to associate with T. cruzi and also to stimulate their cytotoxic activity (52,53). Interferon-γ, the T cell product implicated in macrophage activation (54), has also been shown to enhance the capacity of macrophages to take up and kill trypomastigotes (55). Treatment of trypomastigotes with specific antibody enhances their uptake by unstimulated macrophages in vitro (56), but does not modify their intracellular fate (44,56). In contrast, specific antibody will enhance parasite killing by activated macrophages (56).

A similar trypanocidal activity has been seen when human monocyte-derived macrophages were exposed to T. cruzi-stimulated peripheral blood lymphocytes from patients with chronic Chagas' disease (57). Lymphokines generated by stimulating these lymphocytes with antigens unrelated to the parasite or with concanavalin A were also active. In contrast, supernatants collected after incubation of lymphoid cells from normal donors with T. cruzi antigen did not induce parasitocidal activity (57,58).

IV. Mechanisms of phagocyte toxicity

Microbial killing by phagocytic cells involves a multiplicity of mechanisms, all of which are set in motion by two cellular events: degranulation and the respiratory burst. Degranulation is the process of fusion between the primary phagosomes and the lysosomes (59). The lysosomes contain enzymes and other agents that participate in the killing and degradation of ingested microorganisms. Neutrophils present two major types of intracellular granules (50); 1) the azurophil or primary granules, that contain a diverse array of substances, including acid proteases, glycosidases, 5'-nucleotidases, arylsulfatase, neutral proteases, cationic proteins, myeloperoxidases, lysozyme and acid mucopolysaccharides, and 2) the specific or secondary granules, that contain lactoferrin, alkaline phosphatase, a vitamin-B_{12}-binding protein, lysozyme and collagenase. This spectrum of enzymes is enough to degrade many of the diverse lipids, polysaccharides, and proteins present in microbes.

Macrophages and monocytes can also destroy ingested microorganisms. Qualitatively, the spectrum of proteolytic enzymes produced by macrophages shows similarities with that of neutrophils although there may be differences in the level of the activity of certain enzymes (61). Macrophages can compensate for this deficiency by increasing protein production upon differentiation, especially when stimulated (60).

Another mechanism of killing used by phagocytes is the respiratory burst, a metabolic pathway dormant in resting cells (62) whose function is to produce a group of highly reactive microbicidal agents by the partial reduction of oxygen. The first event in this

pathway is an increase in oxygen uptake (63) upon stimulation of the phagocyte. Stimulation of phagocytosis has also been shown to cause an increase in glucose oxidation via the hexose monophosphate shunt (63), a metabolic pathway in which glucose is oxidized to carbon dioxide and a five carbon sugar with $NADPH^+$ serving as electron acceptor. NADPH oxidase has been implicated as one of the components of the membrane complex that reduces O_2 to $O_2^{\cdot-}$, a metabolite associated with phagocytosis by neutrophils (64). In addition to NADPH oxidase, cytochrome b_{559} (65) and ubiquinone (66) are also believed to be a part of this complex. Nonactivated macrophages have a weaker respiratory burst than granulocytes, but can be immunologically activated, a phenomenon that increases the affinity of the NADPH oxidase for NADPH and brings the activity of the enzyme to levels comparable to that of neutrophils (67).

The first product of the reduction of O_2, $O_2^{\cdot-}$, is dismutated spontaneously to H_2O_2, a reaction that occurs primarily at low pH. The reaction may also be catalyzed by superoxide dismutase at pH 7.0 (68). H_2O_2 is a well known toxic agent (69), whose action can be potentiated by two different mechanisms: peroxidation, leading to the production of hypohalous acids, and the Fenton reaction, which generates $OH^{\cdot}$ and $^1O_2$. In the first of these mechanisms, H_2O_2, halide, and myeloperoxidase, present in the granules of the neutrophils, eosinophils and monocytes, react to form hypohalous acids of which hypochlorous acid is the most common due to the availability of Cl^- in cells. These compounds are very toxic to microorganisms via halogenation and oxidation of cell surface components (68). Macrophage granules are devoid of this peroxidase, possibly having

lost this enzyme during the process of monocyte differentiation (60). Macrophages, however, can also acquire it by endocytosis as has been shown in the killing of eosinophil peroxidase-coated Trypanosoma cruzi and Toxoplasma gondii by resident peritoneal macrophages (70,71).

In the Fenton reaction, H_2O_2 can form OH• radicals and $^1O_2$ in the presence of Fe^{++} as illustrated below:

$$H_2O_2 + Fe^{++} \longrightarrow Fe^{+++} + OH^- + OH^\bullet + {}^1O_2$$

or, if Fe^{+++} is present,

$$Fe^{+++} + O_2^{\bar{\bullet}} \longrightarrow Fe^{++} + O_2$$

$$Fe^{++} + H_2O_2 \longrightarrow Fe^{+++} + OH^- + OH^\bullet + {}^1O_2$$

The latter is the Haber-Weiss reaction and also requires the presence of $O_2^{\bar{\bullet}}$. Neutrophils, monocytes and macrophages have been shown to produce OH• (68,71,142). Whether $^1O_2$ is produced in vivo is not yet clear, though the killing of Toxoplasma gondii by macrophages (72,73) and by the enzymatic system, xanthine-xanthine oxidase (which produces these latter metabolites in vitro) (74), has been inhibited by scavengers of $^1O_2$.

The toxicity of OH• is partly due to its ability to react with membrane lipids by hydrogen atom abstraction, forming a peroxy radical. This radical can in turn, affect adjacent lipids in the membrane (75). Hydroxyl radical can also attack DNA and proteins forming the same peroxy radical in a chain reaction. Recently, it has been shown that iron is involved in the decomposition of lipid peroxides to peroxy radicals, thus amplifying the reaction and contributing to the membrane damage (75).

The role of oxygen metabolites in the killing of T. cruzi by phagocytic cells is suggested by the parasite's inability to detoxify these metabolites. Catalase, an enzyme which decomposes H_2O_2, is absent in the parasite (76) and glutathione peroxidase levels are low (77). A cyanide-sensitive superoxide dismutase has been reported in T. cruzi (78) and a cyanide-insensitive but H_2O_2 and azide-sensitive superoxide dismutase has also been found (79). In agreement with the presence of superoxide dismutases and the lack of an efficient mechanism to degrade H_2O_2, T. cruzi is found to be susceptible to the toxic effects of H_2O_2. Trypomastigotes and amastigotes have been found to be sensitive to H_2O_2 whether enzymatically generated (35,80,81) or added as a reagent (35). Lymphokine- and IFN-stimulated macrophages kill T. cruzi by mechanisms involving H_2O_2 (55,80). Coating T. cruzi with eosinophil peroxidase enables macrophages to kill trypomastigotes in a process inhibitable by both cyanide and catalase (70). Killing of amastigotes by unstimulated neutrophils and macrophages was also shown to be due to H_2O_2 (35,46). T. cruzi is not the only parasite susceptible to these metabolites since the mechanism of killing by activated macrophages of two other intracellular pathogens, Leishmania donovani and Toxoplasma gondii, was also found to be mediated by these compounds (82).

V. Lactoferrin: general considerations

Lactoferrin, present in the specific granules of neutrophils (83), is an iron-binding glycoprotein with a molecular weight of 76 kd (84,85). It was first described in milk (86) and is primarily found in various secretions (87) such as tears, saliva, nasal and bronchial secretions, urine, seminal fluid, and cervical mucus. Although

lactoferrin is found in different types of exudates, two main cell types produce it: epithelial cells in glandular tissue and myelocytes of the granulocytic series in the bone marrow (88). Lactoferrin isolated from a variety of different sources appears to be the same protein (89,90).

Transferrin, an iron binding molecule found primarily in serum, has a significant amino acid homology with lactoferrin (91), but these two proteins do not cross react immunologically (92) and bind to different receptors on cells (92,93).

In the presence of one mole of bicarbonate, lactoferrin binds two moles of Fe^{+++} per mole of protein (94,95). However, the glycoprotein has also been found to form complexes with other metals such as chromium, manganese, cobalt (96), zinc (97), and copper (95). Lactoferrin binds Fe^{+++} with an affinity that, at physiological pH, is 260 times greater than that of transferrin (94). The binding of Fe^{+++} to lactoferrin is stable to low pH (4.0), whereas transferrin releases its Fe^{+++} at pH 5.7 (94).

The concentration of lactoferrin in serum ranges from 0.1 to 1.5 μg/ml in normal non-inflammatory states (98,99). It rises at the onset of inflammation and is increased in the cerebrospinal fluid of patients with meningitis (100,101) or cerebrovascular insults (102) and in the plasma of individuals with acute bacterial infections (103,104). Burn patients also have higher levels of lactoferrin in their plasma (10-40 μg/ml) (105). In burn patients with complicating bacterial infections, lactoferrin values are higher than in burn patients without infections (106). Lactoferrin levels are also increased in the plasma of cystic fibrosis patients suffering from

acute inflammation of the lungs (107) and in rheumatoid states (108). This has been correlated to an increase in degranulation of polymorphonuclear cells *in situ*. Patients with Sjogren's syndrome, an autoimmune disease characterized by chronic inflammation of unknown cause, also have increased levels of lactoferrin in their saliva (109). This is thought to represent increased synthesis of the protein by glandular cells since saliva contains only a few neutrophils.

In the presence of zymosan and *Staphylococcus albus* (110), latex beads coated with IgG (111), *Escherichia coli* (112) or *Treponema denticola* (113), neutrophils can release up to 90% of their granular contents of lactoferrin (111). Some investigators have correlated the increase in lactoferrin concentration in acute inflammatory states with increased neutrophil turnover or greater numbers of neutrophils in the blood (114). In keeping with this hypothesis is the observation that in patients with spontaneous or chemotherapeutically-induced neutropenia, plasma levels of lactoferrin are lower than normal (115,116). These levels increase in patients that go into remission after treatment, and are noticed before concomitant increases in neutrophil counts are achieved (47).

Unlike transferrin, plasma levels of lactoferrin do not correlate well with iron storage in the host since there is no correlation between lactoferrin concentration in iron-deficient subjects and in those who are iron replete (118). Van Snick et al (110) reported the involvement of lactoferrin in the hyposideremia of inflammation. They observed that neutrophils release apolactoferrin upon zymosan-induced phygocytosis. The released lactoferrin was able to bind iron to

saturation, indicating that it had been released from the cell iron-free. The Fe-lactoferrin was then shown to bind monocytes. In vivo studies showed that apolactoferrin injected into rats can cause a marked decrease in the plasma levels of iron. Lactoferrin was found to accumulate in the reticuloendothelial system. In contrast, when human apotransferrin was injected into rats, it increased iron levels. Endotoxin injected into rats decreases the level of iron in the plasma and simultaneously increases levels of lactoferrin in several organs, mainly the liver and lungs. When ^{59}Fe citrate is injected 30 minutes before the endotoxin, the isotope is found to be associated with lactoferrin extracted from these organs. These findings suggest a possible mechanism by which iron is accumulated in the reticuloendothelial system during inflammation. It is possible that this is the same mechanism by which iron is made unavailable to microorganisms in the blood, depriving them of this metal essential for their growth.

A bacteriostatic effect was, in fact, one of the first physiological functions attributed to iron-depleted lactoferrin (119-123). The antimicrobial ability of apolactoferrin was reversed when an excess of iron was supplied to the nutritionally-deprived microorganisms. Apolactoferrin has also been found to be bactericidal for some species of bacteria: Streptococcus mutans and Vibrio cholera (124), Pseudomonas aeruginosa and Escherichia coli (125), and Legionella pneumophila (126). The antimicrobial activity of lactoferrin was verified by the inability of bacteria to grow when transferred to iron-rich medium (124). Consistent with the protective effect of lactoferrin are the findings that two patients suffering

from recurring bacterial infections had severely reduced levels of lactoferrin in their neutrophil granules (127,128).

VI. Lactoferrin: cell binding and its consequences

Several cells of the immune system have receptors for lactoferrin on their surface and the engagement of the ligand in each of the cells leads to different biological effects. Mouse peritoneal macrophages have $2x10^7$ receptors/cell with a Ka of $9x10^5$L/mole (92) as determined by Scatchard plots of the binding of ^{125}I-labeled lactoferrin. The binding is specific and cannot be inhibited by transferrin, although the latter protein also binds to these cells (129). Human monocytes have a slightly higher receptor density ($2x10^8$/cell) with a comparable affinity constant of 3.75×10^5L/mole. The binding is specific and is inhibited by ethylenediaminotetraacetic acid and mannose (93).

The binding of lactoferrin to monocytes has been described to increase their cytotoxic activity for tumor cells (130). Both 20% Fe-saturated and 100% Fe-saturated lactoferrin were effective, whereas transferrin was not. The binding of lactoferrin to monocytes and macrophages has a potent inhibitory activity on granulopoiesis *in vitro* and *in vivo* (131). Eight percent Fe-saturated lactoferrin is inhibitory at 10^{-13}M, whereas 100% Fe-saturated is inhibitory at up to 10^{-17}M. Lactoferrin acts on an Ia bearing subpopulation of macrophages responsible for the production of colony stimulating factor (132). The inhibitory effect is complex since lactoferrin inhibits release of prostaglandin E_2 and acidic isoferritins from macrophages. These molecules are in themselves inhibitory for granulopoiesis (133,134,135). Transferrin, in its iron-saturated form, has no effect on the release of granulopoietic factors from

macrophages, although it suppresses the release of granulocyte macrophage colony stimulating factor from $OKT4^+$ T lymphocyte subpopulations (136). However, the relevance of these observations *in vivo* has been recently questioned (137,138).

Lactoferrin also binds to the surface of neutrophils following secretion from the lysosomal granules (139). The binding of lactoferrin to the neutrophil surface promotes their adhesiveness to each other and to endothelial cells. In one study, neutrophils were found to contain 1.35×10^6 receptors/cell with a Ka of 5.2×10^6 L/mole (140), whereas in another study two kinds of binding sites were demonstrated: a low capacity and high affinity site (3.9×10^4/cell with a Ka of 2.2×10^9 L/mole) and a higher capacity and low affinity site (7.2×10^4/cell with a Ka of 6×10^8 L/mole) (141). This discrepancy remains to be explained.

VII. Lactoferrin: its role in production of oxygen metabolites

Lactoferrin has been shown to be involved in the formation of some reductive oxygen intermediates. It increases $OH^\cdot$ production by human neutrophils, neutrophil particulate fractions and the xanthine-xanthine oxidase enzymatic system (142). This observation has been confirmed by some laboratories (143,144) and denied by another (145). Lactoferrin has also been shown to promote red blood cell lysis by activated neutrophils (146). While agranular neutrophils were unable to lyse the target cells, they were able to generate the same amounts of O_2^- as intact cells. Supplementing agranular cells with lactoferrin reverted the lytic capacity to a level comparable with that of intact neurophils, suggesting that lactoferrin had an effect beyond the production of O_2^- in the cells

(146). On the other hand, lactoferrin without iron or at low level of iron saturation (0-4%) has been shown to inhibit lipoperoxidation of bovine brain liposomes initiated by Fe salts. Apotransferrin was equally effective (147).

The physiological functions of lactoferrin are far from being completely defined. Because of its ability to tightly bind iron and its low concentration in serum, this glycoprotein does not appear to serve as an iron transport protein. Upon binding to receptors in the membrane of several cell types, it induces different types of cellular activities that appear to be contradictory. It is evident that much more must be studied about this protein to ascribe it a specific role in biological processes.

VIII. Research goals

A heavy inflammatory cell infiltrate is found in acute chagasic lesions. These cells are prone to be continuously stimulated *in situ* by several factors, such as by parasites, as they are released from bursting infected cells, and by products released from the inflammatory cells themselves as they come into contact with the parasites. Lactoferrin, a protein secreted by stimulated neutrophils during inflammation, is one of these products and might therefore modulate the activities of other cells found in the lesion.

The focus of this work was to define any modulatory effect that lactoferrin might have on the interaction of phagocytic cells and mammalian forms of *T. cruzi* and to examine the relevant mechanisms.

The first chapter describes the outcome of *in vitro* interaction between mouse peritoneal macrophages or human monocytes and the intracellular amastigote forms of *Trypanosoma cruzi* in the presence of

lactoferrin. The possible similarities and differences in the fate of this form and that of bloodstream trypomastigotes were explored in Chapter II.

Since lactoferrin is an iron binding molecule, the contribution of this metal to lactoferrin's effects on macrophage-parasite interactions was investigated by selectively removing and adding iron to lactoferrin preparations. The results of these studies are presented in Chapter III.

The possibility that lactoferrin might be found *in vivo* in the course of *T. cruzi* infection was studied next, using a mouse model. These results are presented in the Appendix section.

Closing this thesis, a summary will highlight the conclusions derived from this work, their significance and perspectives.

REFERENCES

1. Chagas, C. 1909. Nova tripanosomiase humana. Estudos sobre a morfologia e o ciclo evolutivo do Schizotrypanum cruzi, h. gen., n. op., agente etiologico da nova entidade morbida do homem. Mem Inst. Oswaldo Cruz: 1:159-218.

2. Schiffler, R.J., Mansur, G.P., Navin, T.R., and Limpakarnianarat, K. 1983.Indigenous Chagas; Disease (American trypanosomiasis) in California. J. Am. Med. Assn. 25:2983-2984.

3. Navin, T.R., Roberto, R.R., Juranek, D.D., Loimpakarnjanarat, K., Mortenson, E.W., Clover, J.R., Yescott, R.E, Taclindo, C., Steurer, F., and Allain, D. 1985. Human and sylvatic Trypanosoma cruzi infection in California. Am. J. Publ. Hlth. 75:366-369.

4. Amato-Neto, V. 1979. Transmissao por transfusao de sangue. Congresso Internacional sobre Doenca de Chagas. Rio de Janeiro, page 421.

5. Science at work. (1984) UNDP/World Bank/WHO, Geneva.

6. Gomez Pereira, M. 1984. Caracteristicas da mortalidade urbana por Doenca de Chagas, Distrito Federal, Brazil. Boletin da Oficina Sanitaria Pan Americana 96:213-221.

7. Laranja, F.S. 1953. Aspectos Clinicos da molestia de Chagas. Rev Bras Med 10:482-491.

8. Chagas, C. 1916. Tripanosomiase Americana: forma aguda da molestia. Mem. Inst. Oswaldo Cruz 8:15-36.

9. Rassi, A. 1979. Clinica: Fase aguda. In Trypanosoma cruzi e doenca de Chagas. p. 249-264. Z. Brener and Z. Andrade eds. Guanabara Koogan, Rio de Janeiro.

10. Andrade, Z. 1985. A Patologia da doenca de Chagas no homem. Ann. Soc. Belge. Med. Trop. 65 Suppl 1:15-30.

11. Rezende, J.M. 1979. Clinica:manifestacoes digestivas. In Trypanosoma cruzi e Doenca de Chagas. p. 312-361. Z Brener and Z. Andrade eds. Guanabara Koogan, Rio de Janeiro.

12. Vianna, G. 1911. Contribuicao para o estudo da anatomia patologica da Molestia de Carlos Chagas. Mem. Inst. Oswaldo Cruz 3:276-293.

13. Romana, C. 1943. Contribuicao ao conhecimento da patogenia da tripanosomiase Americana. Mem. Inst. Oswaldo Cruz 39:253-264.

14. Andrade, Z. A. and Andrade, S.G. 1963. Forma subaguda da miocardite chagasica. Rev. Inst. Med. Trop. S. Paulo 5:273-280.

15. Andrade, Z., and Andrade, S.G. 1979. Patologia. In Trypanosoma cruzi e Doenca de Chagas. p. 199-248. Z. Brener and Z. Andrade eds. Guanabara Koogan, Rio de Janeiro.

16. Herriquez, D., Piras, R.M., and Piras, M.M. 1981. The effect of surface membrane modification of fibroblastic cells on the entry process of Trypanosoma cruzi trypomastigotes. Mol. Bioch. Parasitol. 2:359-366.

17. Villalta, F., and Kierszenbaum, F. 1983. Role of cell surface mannose residues in host cell invasion by Trypanosoma cruzi. Biochim. Biophys. Acta. 736:39-44.

18. Villalta, F. and Kierszenbaum. F. 1984. Host-cell invasion by Trypanosoma cruzi: role of cell surface galactose residues. Biochem. Biophys. Res. Common. 119:228-235.

19. Villalta, F. and Kierszenbaum, F. 1985. Role of surface N-acetylglucosamine residues on host cell infection by Trypanosoma cruzi. Biochem. Biophys. Acta. 895:216-222.

20. Piras, R.M., Piras, M.M., and Henriquez, D. 1983. Trypanosoma cruzi fibroblastic cell interaction necessary for cellular invasion. In Cytopathology of parasitic disease. p. 31-55. Pitman, London (Ciba foundation symposium 99).

21. Lima, M.F. and Kierszenbaum, F. 1982. Biochemical requirements for intracellular invasion by Trypanosoma cruzi: protein synthesis. J. Protozool. 29:566-570.

22. Lima, M.F. and Kierszenbaum, F. 1983. Effects of treatment of trypomastigote forms of Trypanosoma cruzi or host cells with ethidium bromide on cell infection and intracellular fate of the parasite. J. Parasitol. 70:911-917.

23. Villalta, F. and Kierszenbaum, F. 1985. The effect of swainsonine on the association of Trypanosoma cruzi with host cells. Mol. Biochem. Parasitol 16:1-10.

24. Alves, M.J.M., Colli, W. 1974. Agglutination of Trypanosoma cruzi by concanavalin A. J. Protozool. 21:575-578.

25. Pereira, M.E.A., Loures, M.A., Villalta, F., and Andrade, A.F.B. 1980. Lectin receptors as markers for Trypanosoma cruzi. J. Exp. Med. 152: 1375-1392.

26. Araujo, F.G., Handman, E., Remington, J.S. 1980. Binding of lectins to cell surface of Trypanosoma cruzi. J. Protozool. 27:397-400.

27. Crane, M.S.J., and Dvorak, J.A. 1982. Influence of monosaccharides on the infection of vertebrate cells by Trypanosoma cruzi and Toxoplasma gondii. Mol. Biochem. Parasitol. 5:333-341.

28. Colli, W., Katzin, A.M., Andrews, N.W., Kuwajima, V.Y., Goncalves, M.F., Abuin, G., Alves, M.J.M., Zingales, B. 1984. Perspectives of vaccination against Chagas' disease through biotechnology. I. Definition of relevant antigens possibly involved in parasite-host-cell interaction. In New Approaches to vacine development. p. 418-441. Bell, R., Torrigiani, G. (eds). Schwabe, Basel.

29. Snary, D., Hudson, L. 1979. Trypanosoma cruzi cell surface proteins: identification of one major glycoprotein. FEBS letters 100:166-170.

30. Scott, M.T., and Snary, D. 1979. Protective immunization of mice using cell surface glycoprotein from Trypanosoma cruzi. Nature 282:73-74.

31. Snary, D. 1983. Cell surface glycoproteins of Trypanosoma cruzi: protective immunity in mice and antibody levels in human Chagasic sera. Trans. R. Soc. Trop. Med. Hyg. 77:126-129.

32. Katzin and Colli, W. 1983. Lectin receptors in Trypanosoma cruzi, an N acetyl D glucosamine containing surface glycoprotein specific for the trypomastigote stage. Biochim Biophys Acta 727:403-411.

33. Zingales, B., and Colli, Z. 1985. Trypanosoma cruzi: interaction with host cells. Current Topics in Microbiol. and Immunol. 117:129-148.

34. Peterson, D.S., Wrightsman, R.A., Manning, J.E. 1986. Cloning of a major surface antigen gene of Trypanosoma cruzi and identification of a nonapeptide repeat. Nature 322:566-568.

35. Villalta, F., and Kierszenbaum, F. 1983. Role of polymorphonuclear cells in Chagas' disease. I. Uptake and mechanism of destruction of intracellular (amastigote) forms of Trypanosoma cruzi by human neutrophils. J. Immunol. 131:1-7.

36. Olabuenaga, S.E., Cardoni, R.L., Segura, E.L., Riera, N.E., and De Bracco, M.M.E. 1979. Antibody-dependent cytolysis of Trypanosoma cruzi by human polymorphonuclear leukocytes. Cell. Immunol. 45:85-93.

37. Madeira, E.D., De Andrade, A.F.B., Bunn-Moreno, M.H., and Barcinski. M. 1979. Antibody dependent cellular cytotoxicity of Trypanosoma cruzi: characteristics of the effect of cells from blood. Infect. Immun. 25:34-38.

38. Cardoni, R.L., do Campo, R., and Casellas, A.M. 1982. Metabolic requirements for the damage of Trypanosoma cruzi epimastigotes by human polymorphonuclear leukocytes. J. Parasitol. 68:547-552.

39. Kierszenbaum, F. 1979. Antibody dependent killing of blood-stream forms of Trypanosoma cruzi by human peripheral blood leukocytes. Am. J. Trop. Med. Hyg. 28:965-968.

40. Okabe, K., Kipnis, T.L., Cauch, V.L. and Dias da Silva, W. 1980. Cell mediated cytotoxicity to Trypanosoma cruzi: infection. I. Antibody dependent cell mediated cytotoxicity to trypomastigote bloodstream forms. Clin. Immunol. Immunopathol. 16:344-353.

41. Kierszenbaum, F. and Lima, M.F. 1983. Susceptibility of insect-borne, metacyclic forms of Trypanosoma cruzi to antibody mediated mechanisms of destruction. Am. J. Trop. Med. Hyg. 32:1236-1241.

42. do Campo. R., Casellas, A.M., Madeira, E.D., Cardoni, R.L. , Moreno, S.N.J., and Mason, R.P. 1983. Oxygen-derived radicals from Trypanosoma cruzi stimulated human neutrophils. FEBS Letters 153:25-30.

43. Villalta, F., and Kierszenbaum, F. 1983. Role of inflammatory cells in Chagas' disease. I. Uptake and mechanisms of destruction of intracellular (amastigote) forms of Trypanosoma cruzi by human eosinophils. J. Immunol. 132:205-2058.

44. Nogueira, N., and Cohn, Z. 1976. Trypanosoma cruzi: mechanisms of entry and intracellular fate in mammalian cells. J. Exp. Med. 143:1402-1420.

45. Kipnis, T.L., Calich, V.L.G., and Da Silva, W.D. 1979. Active entry of bloodstream forms of Trypanosoma cruzi into macrophages. Parasitol. 78:89-98.

46. Villalta, F., and Kierszenbaum, F. 1984. Role of inflammatory cells in Chagas' disease. II. Interaction of mouse macrophages and human monocytes with intracellular forms of Trypanosoma cruzi: uptake and mechanism of destruction. J. Immunol. 133:3338-3343.

47. Carvalho, R.M., Meirelles, M.N.L., de Souza, W., and Leon, W. 1981. Isolation of the intracellular stage of Trypanosoma cruzi and its interaction with mouse macrophages in vitro. Infec. Immun. 33:546-554.

48. Wirth, J.J., and Kierszenbaum, F. 1984. Fibronectin enhances macrophage association with invasive forms of Trypanosoma cruzi. J. Immunol. 133:460-464.

49. Ouassi, M.A., Afchain, D., Capron, A., Grimaud, J.A. 1984. Fibronectin receptors on Trypanosoma cruzi trypomastigotes and their biological function. Nature. 308:380-382.

50. Nogueira, N., and cohn, Z. 1978. Trypanosoma cruzi: in vitro induction macrophage microbicidal activity. J. Exp. Med. 198:288-300.

51. Ford-Hutchinson, A.W. 1985. Leukotrienes: their formation and role as inflammatory mediators. Fed. Proceedings 44:25-29.

52. Wirth, J., and Kierszenbaum, F. 1985. Stimulatory effects of leukotriene B_4 on macrophage association with and intracellular destruction of Trypanosoma cruzi. J. Immunol. 134:1989-1993.

53. Wirth, J., and Kierszenbaum, F. 1985. Effects of leukotriene C_4 on macrophage association with and intracellular fate of Trypanosoma cruzi. Mol. Biochem. Parasitol. 15:1-10.

54. Nathan, C.F., 1986. Macrophage activation: some questions. Ann. Inst. Pasteur / Immunol. 137c:345-351.

55. Wirth, J.J., Kierszenbaum, F., Sonnenfeld, G., and Zlotnik. 1985. Enhancing effects of gamma interferon on phagocytic cell association with and killing of Trypanosoma cruzi. Infect. Immun. 49:61-66.

56. Nogueira, N., Chaplan, S., and Cohn, Z. 1980. Trypanosoma cruzi: factors modifying ingestion and fate of blood forms by macrophages. J. Exp. Med. 152:447-451.

57. Nogueira, N., Chaplan, S., Rusink, M., Tydings, J., and Cohn, Z. 1982. Trypanosoma cruzi: induction of microbicidal activity in human mononuclear phagocytes. J. Immunol. 128:2142-2146.

58. Williams, D.M., and Remington, J.S. 1977. Effects of human monocytes and macrophages on T. cruzi. Immunology 32:19-23.

59. Zucher-Franklin, D., Hirsch, J.G. 1964. Electron microscope studies on the degranulation of rabbit peritoneal leukocytes during phagocytosis. J. Exp. Med. 120:569-675.

60. Parker, C.W. 1984. Mediators: release and function. In Fundamental Immunology, p. 697-747. W.E. Paul, ed. Raven Press, New York.

61. Hughes, K.T., Coles, G.A., Harry, T.R., and Davies, M. 1981. Some properties of human blood monocytes cell lysate neutral proteinases. Biochem. Biophys. Acta 662:111-118.

62. Sbarra, A.J., Karnovsky, M.L. 1959. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. J. Biol. Chem. 234:1355-1362.

63. Iyer, G.Y.N., Islam, M.F., Quastel, J.H. 1971. Biochemical aspects of phagocytosis. Nature 192:535-541.

64. Babior, B.M., Kipnes, R.S. Curnutte, J.T. 1973. Biological defense mechanisms, the production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. 52:741-744.

65. Segal, A.N., Jones, O.T.G. 1978. Novel cytochrome b system in phagocytic vacuoles of human granulocytes. Nature 276:515-517.

66. Crawford, D.R., Scheineder, D.L. 1983. Ubiquinone content and respiratory burst activity of latex-filled phagolysosomes isolated from human neutrophils and evidence for the probable involvement of a third granule. J. Biol. Chem. 258:5363-5367.

67. Tsunawaki, S., Nathan, C.F. 1984. Enzymatic basis of macrophage activation. J. Biol. Chem. 259:4305-4312.

68. Klebanoff, S. 1982. Oxygen dependent cytotoxic mechanisms of phagocytes. In Advances of Host Defense Mechanisms. Vol. 1:111-157. John I. Gallin and A.S. Fauci, eds. Raven Press, N.Y.

69. Klebanoff, S.S., Lockesley, R.M., Jong, E.C., Rosen, H. 1983. Oxidative response of phagocytes to parasite invasion. In Cytopathology of parasitic disease. p. 92-112. Pitman Books, London, (CIBA Foundation Symposium, 99).

70. Locksley, R.M., Wilson, C.B., Klebanoff, S.J. 1982. Role of endogenous and acquired peroxidase in the toxoplasmacidal activity of murine and human mononuclear phagocytes. J. Clin. Invest. 69:1099-1111.

71. Nogueira, N., Klebanoff, S., and Cohn, Z.A. 1982. T. cruzi: sensitization to macrophage killing by eosinophil peroxidase. J. Immunol. 128:1705-1708.

72. Murray, H.W., Juangbhaanich, C.W., Nathan, C., Cohn, Z.A. 1979. Macrophage dependent antimicrobial activity: The role of oxygen intermediates. J. Exp. Med. 150:950-964.

73. Murray, H.W., Conh, Z.A. Macrophage oxygen dependent antimicrobial activity III: enhanced oxidative metabolism as an expression of macrophage activation. J. Exp. Med. 152:1596-1609.

74. Murray, H.N., Cohn, Z.A. 1979. Macrophage oxygen-dependent antimicrobial activity I: susceptibility of Toxoplasma gondii to oxygen intermediates. J. Exp. Med. 150:938-949.

75. Hallwell, B., Gutteridge, J.M.C. 1986. Iron and free radical reactions: two aspects of antioxidant protection. Trends Biochem Sci. 11:374-375.

76. do Campo, R., de Boiso, J.F., Boveris, A., Stoppani, A.D.M. 1976. Localization of peroxidase activity in Trypanosoma cruzi microbodies. Experientia 33:972-975.

77. Boveris, A., Sies, H., Martino, E.E., Docampo, R., Turrens, J.F., Stoppani, A.D.M. 1980. Deficient metabolic utilization of hydrogen peroxide in Trypanosoma cruzi. Biochem. J. 188:643-648.

78. Boveris, A., Stoppani, A.O.M. 1977. Hydrogen peroxide generation in Trypanosoma cruzi. Experientia 33:1306-1308.

79. Meshnick. S.R., Trang, N.O., Kitchener, K., Cerami, A., Eaton, J. 1983. Iron containing superoxide dismutase in trypanosomatids. In: Cohen, G., Greenwalk, R.A. eds. p. 348-351. Oxy-radicals and their scavenger systems; molecular aspects. Vol. I Elsevier-North Holland, New York.

80. Nathan, C.F., Nogueira, N., Juangbhaanic, C., Ellis, J., Cohn, Z.A. 1979. Activation of macrophages in vivo and in vitro. Correlation between hydrogen peroxide release and killing of Trypanosoma cruzi. J. Exp. Med. 149:1056-1068.

81. Tonaka, Y., Tanowitz, H., Bloom, B.R. 1983. Growth of Trypanosoma cruzi in a cloned macrophage cell line and in a variant defective in oxygen metabolism. Infect. Immun. 41:1322-1331.

82. Murray, H. 1984. Macrophage activation: enhanced oxidative and antiprotozool activity. Contemporary topics in Immunobiology. vol. 13:97-115. D.O. Adams, M.G. Hanna, eds. Plenum Press, New York.

83. Masson, P.L., Heremans, J.F., and Schonne, E. 1969. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. J. Exp. Med. 130:643-658.

84. Castellino, F.J., Fish, W.F., Mann, K.G. 1970. Structural studies on bovine lactoferrin. J. Biol. Chem. 245:4269-4275.

85. Querinjean, P., Masson, P.L., Heremans, J.F. 1981. Molecular weight, single chain structure and amino acid composition of human lactoferrin. Eur. J. Biochem. 20:420-425.

86. Sorensen, M., and Sorensen, S.P.L. 1939. The proteins in whey. Compt. Rend. Trav. Lab. Calsberg 23:55-59.

87. Masson, P.L., Heremans, J.F. 1966. Studies on lactoferrin, the iron binding protein of secretions. Protides Biol. Fluids Proc. Colloq. Bruges 19:115-124.

88. Rado, T.A., Bollekens, J., St. Laurent, G., Parker, L., Benz, E.J. 1984. Lactoferrin biosynthesis during granulopoiesis. Blood 64:1103-1109.

89. Moquilevsky, N., Retegui, L.A., Masson, P.L. 1985. Comparison of human lactoferrins from milk and neurophilic leukocytes. Relative molecular mass, isoelectric point, iron-binding properties and uptake by the liver. Biochem. J. 229:353-359.

90. Wang, C.S., Chan, W.Y., Kloer, H.U., 1984. Comparative studies on the chemical and immunochemical properties of human milk, human pancreatic juice and bovine milk lactoferrin. Comp. Bioch. Physiol. 7813:575-580.

91. Bluard-Deconinck, J., Masson, P.L., Osinski, P.A., Heremanns, J.F. 1974. Amino acid sequence of cystic peptides of lactoferrin and demonstration of similarities between lactoferrin and transferrin. Biochem. Biophys. Acta. 365:311-.

92. Van Snick, J.L., Masson, P.L. 1976. The binding of human lactoferrin to mouse peritoneal cells. J. Exp. Med. 144:1568-1580.

93. Bennet, R.M., Davis, J. 1981. Lactoferrin binding to human peripheral blood cells: an interaction with a β-enriched population of lymphocytes and a subpopulation of adherent mononuclear cells. J. Immunol. 127:1211-1216.

94. Aisen, P., and Leibman, A. 1972. Lactoferrin and transferrin: a comparative study. Biochim. Biophys. Acta 257:314-232.

95. Masson, P.L. and Heremans, S.F. 1968. Metal combining properties of human lactoferrin (red milk protein) I. The involvement of iron in the reaction. Eur. J. Biochem. 6:579-584.

96. Ainscough, E.W., Brodie, A.M., Plowman, J.E. 1979. The chromium, manganese, cobalt and copper complexes of human lactoferrin. Chim. Acta 33:149-153.

97. Ainscough, E.W., Brodie, A.M., Plowman, J.E. 1980. Zinc transport by lactoferrin in human milk. Am. J. Clin. Nutr. 33:1314-1315.

98. Birgens, H.S. 1985. Lactoferrin in plasma measured by an ELISA technique: evidence that plasma lactoferrin is an indicator of neutrophil turnover and bone marrow activity in acute leukemia. Scand. J. Haematol. 34:326-331.

99. Chung, S., Hayward, C., Brock, D.J.H., and Heyningen, V.V. 1985. A monoclonal antibody-based immunoassay for human lactoferrin. J. Immunol. Meths. 84:135-141.

100. Hallgreen, R., Trent, A., Vinge, P. 1982. Lactoferrin, lyzozyme and α- 2 microglobulin levels in cerebrospinal fluid. Differential indices of CNS inflammation. Inflammation 6:291-304.

101. Gutteberg, J.G., Flegstad, T., Jorgensen, T., 1986. Lactoferrin, C-reactive protein, anti-trypsin and immunoglobulin G in cerebrospinal fluid in meningitis. Acta Paediar. Scand. 75:569-572.

102. Terent, A., Hallgren, R., Venge, P., Bergstrom, K. 1981. Lactoferrin, lysozyme and α-2 microglobulin in cerebrospinal fuid. Elevated levels in patients with acute cerebrovascular lesions as indices of inflammation. Stroke 12:40-46.

103. Hansen, N.E., Karle, H., Andersen, V., Malmquist, S., Hoff, G.E. 1976. Neutrophilic granulocytes in acute bacterial infection. Clin. Exp. Immunol. 26:463-468.

104. Gutteberg, T.J., Haneberg, B., Jorgensen, T. 1984. The latency of serum acute phase proteins in meningococcal septicemia, with special emphasis on lactoferrin. Clin. Chim. Acta 136:173-178.

105. Wolach, B., Coates, T.D., Hugli, T.E., Baehner, R.L. Boxer, L.A. 1984. Plasma lactoferrin reflects granulocyte activation via complement in burn patients. J. Lab. Clin. Med. 103:284-293.

106. Chiarelli, A., Casadei, A., Andrian, C., Ongaro, G., Mazzoleni, F. 1985. Lactoferrin serum levels in burned patients. Boll. Inst. Sietoter. Milan 64:86-88.

107. Benini, L., Agostini, M., Vaona, B., Brocco, G., Vantini, I., Cavallini, G., Mostella, G. Sauro, L.A. 1985. Plasma levels of lactoferrin in children suffering from cystic fibrosis. Digestion 31:200-204.

108. Bennett, R.M., Skosey, J.L. 1977. Lactoferrin and lysozyme levels in synovial fluid: differential indices of articular inflammation and degradation. Arthritis Rheum 20:84-90.

109. Kronttinen, Y.T., Kulomaa, M., Mallmstrom, M., Kilpi, A., Reitamo, S. 1984. Lactoferrin in Sjogren's syndrome. Arthritis Rheum. 27:462-467.

110. Van Snick, J., Masson, P.L., Heremans, J. 1974. The involvement of lactoferrin in the hyposideremia of acute inflammation. J. Exp. Med. 140:1068-1084.

111. Leffel, M., Spitznagel, J. 1975. Fate of human lactoferrin and myeloperoxidase in phagocytizing human neutrophils: effects of immunoglobulin G subclasses and immune complexes coated on latex beads. Infect Immun. 12:813-820.

112. Pryzwanski, K.B., MacRae, G.K., Spitznagel, J.K., Cooney, M.H. 1979. Early degranulation of human neutrophils: immunocytochemical studies of surface and intracellular phagocytic events. Cell 18:1025-1033.

113. Hurlen, B., Olsen, I., Lingaas, E., Midvedt, T. 1984. Neutrophil phagocytosis of Treponema denticola as indicated by extracellular release of lactoferrin. Acta Path. Microbiol. Immunol. Scand. Sect. B 92:171-173.

114. Hansen, N.E., Malmquist, J., Thorell, J. 1975. Plasma myeloperoxidase and lactoferrin measured by radioimmunoassay: relation to neutrophil kinetics. Acta Med Scand 198:437-443.

115. Bennett, R.M., Mohla, C. 1976. A solid-phase radioimmunoassay for the measurementof lactoferrin in human plasma: variations with age, sex and disease. J. Lab. Clin. Med. 88:156-166.

116. Oloffson, T., Olsson, I., Benge, P., Elgefors, B. 1977. Serum myeloperoxidase and lactoferrin in neutropenia. Scand. J. Haematol. 18:73-80.

117. Brown, R.D., Rickard, A., Kronenberg, H. 1984. Early detection of granulocyte regeneration after marrow transplantation by plasma lactoferrin. Transplantation. 37:423-424.

118. Buynes, R.D., Bezwoda, W.R., Derman, D.P., Khan, Q., Hansoor, N. 1986. Lack of correlation between iron stores and plasma lactoferrin concentration. Scand. J. Haematol. 36:111-114.

119. Bullen, J.J., Rogers, H.J., Leigh, L. 1972. Iron binding proteins in milk and resistance to Eschrichia coli infection in infants. Brit. Med. J. 1:69-75.

120. Reiter, B., Brock, J.H., Steel, E.D. 1975. Inhibition of Escherichia coli by bovine colostrum and post-colostral milk. II. The bacteriostatic effect of lactoferrin on a serum susceptible and a serum resistant strain of E. coli. Immunology 28:83-95.

121. Oram, J.D., Reiter, B. 1968. Inhibition of bacteria by lactoferrin and other chelating agents. Biochem. Biophys. Acta 170:351-365.

122. Reiter, B., Oram, J.D. 1968. Bacterial inhibitors in milk and other biological fluids. Nature (London) 216:328-.

123. Kirkpatrick, C.H., Gran, R.R., Rich, A.L., Schade, J. 1971. Inhibition of growth of Candida albicans by iron unsaturated lactoferrin: relation to host defense mechanisms in chronic mucocutaneous candidiasis. J. Inf. Dis. 124:539.

124. Arnold R., Cole, M.F., McGhee. 1977. A bactericidal effect for human lactoferrin. Science 197:263-265.

125. Arnold, R.R., Brewer, M., Gauthier, J.J. 1980. Bactericidal activity of human lactoferrin: sensitivity of a variety of microorganisms. Infect. Immun. 28:893-898.

126. Bortner, C.A., Miller, R.D., Arnold, R. 1986. Bactericidal effect of lactoferrin on Legionella pneumophila. Infect Immun. 51:373-377.

127. Breton-Grorius, J., Mason, D.Y., Buriot, D., Vilde, J.L. Griscelli, C. 1980. Lactoferrin deficiency as a consequence of lack of specific granules in neutrophils from a patient with recurrent infections. Am. J. Pathol. 99:413-419.

128. Boxer, L.A., Coates, M.D., Haak, R.A., Wolach, J.B., Hoffstein, S., Baehner, R.L. 1982. Lactoferrin deficiency associated with altered granulocyte function. N. Engl. J. Med. 307:404-410.

129. Hamilton, T.A., Weiel, J.E., Adams, D.O. 1984. Expression of the transferrin receptor in murine peritoneal macrophages is modulated in the different stages of activation. J. Immunol. 132-2285-2290.

130. Nishiya, K., Horwitz, D. 1982. Contrasting effects of lactoferrin on human lymphocyte and monocyte natural killer activity and antibody dependent cell mediated cytotoxicity. J. Immunol. 129:2519-2523.

131. Broxmeyer, H.E., Smithyman, A., Eger, R.R., Meyers, P.A., de Sousa, M. 1978. Identification of lactoferrin as the granulocyte-derived inhibitor of colony-stimulating activity production. J. Exp. Med. 198:1052-1067.

132. Broxmeyer, H.E., Platzer, E. 1984. Lactoferrin acts on I-A and I-E/c antigen subpopulations of mouse peritoneal macrophages in the absence of T lymphocytes and other cell types to inhibit production of granulocyte macrophage colony stimulating factors in vivo. J. Immunol. 133:306-314.

133. Pelus, L.M., Broxmeyer, H.E., Moore, M.A.S. 1979. Regulation of macrophage and granulocyte proliferation. Specificities of prostaglandin E and lactoferrin. J. Exp. Med. 150:277-292.

134. Broxmeyer, H.E., Bognacki, J., Dorner, M.H., and de Sousa, M. 1981. The identification of leukemia-associated inhibitory activity (LIA) as acidic isoferritins: a regulatory role for acidic isoferritins in the production of granulocytes and macrophage. J. Exp. Med. 153:1426-1444.

135. Broxmeyer, H.E., Juliano, L., Waheed, A., Shadduck, R. 1985. Release from mouse macrophages of acidic isoferritins that suppress hematopoietic progenitor cells in induced by purified L cell colony stimulating factors and suppressed by human transferrin. J. Immunol. 135:3224-3231.

136. Broxmeyer, H.E., Lu, L., Bognacki, J. 1983. Transferrin derived from an OKT8 positive subpopulation of T lymphocytes suppresses the production of granulocyte - macrophage colony stimulating factors from mitogen activated T lymphocytes. Blood 62:37-50.

137. Poppas, A., Faith, M.R., Bierman, H.R. 1986. In vivo studies of lactoferrin in murine rebound myelopoiesis. Am. J. Hematol. 22:1-8.

138. Ross, D.W. 1986. Lactoferrin as a regulator of hematopoiesis. Blood Cells 11:455-457.

139. Oseas, R., Yang, H.H., Bachner, R.L., Boxer, L.A. 1981. Lactoferrin: a promoter of polymorphonuclear leukocyte adhesiveness. Blood 57:939-945.

140. Boxer, L.A., Haak, R.A., Yang, H.H., Wolach, J.B., Whitcomb, J.A., Butterick, C.J., Baehner, R.L. 1982. Membrane-bound lactoferrin alters the surface properties of polymorphonuclear leukocytes. J. Clin. Invest. 70:1049-1057.

141. Maneva, A., Slrakov, L.B., Manev, V.V. 1983. Lactoferrin binding to neutrophilic polymorphonuclear leucocytes. Int. J. Biochem. 15:981-984.

142. Ambruso, D.R., Johnston, R.B. 1981. Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system. J. Clin. Invest. 67:352-360.

143. Bannister, J.V., Bannister, W.H., Hill, A.D.O., Thornalley. 1982. Enhanced production of hydroxyl radicals by the xanthine-xanthine oxidase reaction in the presence of lactoferrin. Biochim. Biophys. Acta. 715:116-120.

144. Winterbourn, C., 1983. Lactoferrin-catalysed hydroxyl radical production. Additional requirement for a chelating agent. Biochim. J. 240:15-19.

145. Baldwin, D.A., Jenny, E.R., Aisen, P. 1984. The effects of human serum transferrin and milk lactoferrin on hydroxyl radical formation from superoxide and hydrogen peroxide. J. Biol. Chem. 259:13391-13394.

146. Vercellotti, G.M., Van Asbeck, S., Jacob, H.S. 1985. Oxygen radical induced erythrocyte hemolysis by neutrophils. J. Clin. Invest. 76:956-962.

147. Gutteridge, J.M.C., Paterson, S.K. Segal, A.W., Halliwell, B.A. 1981. Inhibition of lipid peroxidation by the iron-binding protein lactoferrin. Biochem J. 199:259-261.

CHAPTER I

LACTOFERRIN EFFECTS ON PHAGOCYTIC CELL FUNCTION. INCREASED UPTAKE AND KILLING OF AN INTRACELLULAR PARASITE BY MURINE MACROPHAGES AND HUMAN MONOCYTES

ABSTRACT

Mouse peritoneal macrophages (MPM) or human blood monocytes (HBM) co-cultured with intracellular (amastigote [AMA]) forms of Trypanosoma cruzi in the presence of human lactoferrin (LF) took up greater numbers of organisms than in the absence of LF; the proportion of phagocytes taking up AMA was also significantly increased. Pretreatment of either MPM or AMA with LF also enhanced cell-parasite association. By immunofluorescence, HBM, MPM and AMA were found bind LF. Using ^{125}I-labelled LF, each AMA was determined to have approximately 1 X 10^6 receptors for LF. The enhancing effect of LF on cell-parasite association was inhibited when either rabbit anti-LF IgG or α-methyl mannoside (α-MM) was present during incubation of MPM or AMA with LF or when AMA pretreated with LF were then incubated with either LF-blocking agent. While these findings seemed to suggest that LF increased MPM-AMA association by bridging these cells, the LF effect was not inhibited when MPM pretreated with LF were subsequently incubated with either α-MM or anti-LF. Furthermore, LF stimulated phagocytosis as denoted by a significant increase in latex particle uptake after LF treatment of MPM. The intracellular killing capacity of HBM or MPM was also stimulated by LF and was denoted by increased AMA destruction after LF treatments. The possibility that LF only appeared to increase the rate of AMA killing by simply promoting the engulfment of greater numbers of AMA that would then be destroyed intracellularly seemed unlikely because untreated MPM that had already taken up untreated AMA killed greater numbers of AMA when they were subsequently incubated with LF. The results

of experiments with scavengers of oxygen reduction intermediates and of nitroblue tetrazolium reduction tests indicated that H_2O_2, O_2^- and $^1O_2$ were involved in the killing of AMA by LF-treated MPM. These results suggest that LF, a glycoprotein secreted by neutrophils in greater-than-normal amounts during inflammation, may contribute to macrophage clearance of AMA released from infected host cells.

INTRODUCTION

Inflammatory cells have been postulated to play a role in the clearance of intracellular (amastigote [AMA]) forms of Trypanosoma cruzi, the etiologic agent of Chagas' disease (1, 2). Thus, unelicited mouse peritoneal macrophages (MPM) (2, 3), human blood monocytes (HBM) (2, 4), human neutrophils (5) and eosinophils (6) have been shown to internalize T. cruzi AMA and destroy these organisms within their phagocytic vacuoles (2, 5, 6). However, our understanding of the mechanisms promoting uptake of microorganisms by inflammatory cells remains deficient. Recent work has identified fibronectin, a plasma protein produced by macrophages (7), as a molecule that can mediate and modulate the uptake of bloodstream forms of T. cruzi and inert particles by unelicited murine MPM (8). This observation and the well recognized accumulation of inflammatory cells in acute chagasic tissue lesions (reviewed in reference 9) have directed our attention towards the possible role played by proteins secreted by inflammatory cells in phagocytic cell interactions with mammalian forms of T. cruzi. Lactoferrin (LF), an iron-binding glycoprotein found in milk, saliva, gastrointestinal fluids and blood (reviewed in reference 10), is produced and secreted in discrete amounts by neutrophils (11, 12) -cells which accumulate at inflammatory chagasic tissue lesions (9)- and its plasma levels increased during inflammatory conditions (13). This information, and the observation that LF increases the natural killer cytotoxicity of adherent monocytes for tumor cells (14), prompted the present study in which we tested the ability of LF to influence the early and subsequent stages of MPM or HBM interaction with T. cruzi AMA.

MATERIALS AND METHODS

Animals. Four- to six-week old Crl:CD-1(ICR)BR Swiss mice were purchased from Charles River Laboratory (Portage, MI).

Parasites. AMA forms of Tulahuén strain T. cruzi were grown in ML-15HA medium as previously described in detail (15). The AMA preparations used in this work consisted of 100% organisms in the AMA form and contained 99 to 100% viable parasites (i.e., organisms displaying their typical vibratilein situ motion). The AMA were washed three times by centrifugation (800 X G,20 min) with Dulbecco's modified minimal essential medium containing 100 µg streptomycin and 100 IU penicillin per ml (DMEM) and finally resuspended in the same medium supplemented with 1% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO) (DMEM+BSA) at 1.25 X 10^7 organisms per ml.

MPM and HBM. Mice sacrificed by exposure to excess ether anesthesia were injected intraperitoneally with 5 ml of sterile DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) (DMEM+FBS) containing 10 U Heparin/ml. The methods for collecting and processing the peritoneal cells and for preparing the resident MPM monolayers have been described (16). The MPM monolayers consisted of >98% nonspecific esterase-positive cells with typical macrophage morphology. Adherent MPM were further incubated under the same conditions (5% CO_2 in air saturated with water vapor) overnight. The methods for collecting and purifying HBM and for setting up the cultures have been described (2). Cultures of these cells consisted of >99% viable, nonspecific esterase-positive cells with typical HBM morphology.

Biological reagents. Batches of human LF (at 10 to 20% iron saturation) were purchased from Sigma. The IgG fraction of rabbit anti-human LF was purchased from Cappel Laboratories (West Chester, PA). Beef erythrocyte superoxide dismutase (SOD) was obtained from Miles Laboratory (Naperville, IL) and beef liver catalase from Sigma. The concentrations at which these reagents were used are described below with the corresponding method.

Measurement of AMA uptake by MPM or HBM. The procedure to measure AMA uptake by MPM has been described (2). Briefly, MPM or HBM monolayers received 0.2 ml of AMA suspension and 0.1 ml of DMEM+BSA, and were incubated at 37°C in a 5% CO_2 incubator for 2 hr. After removing the free AMA by several washings with DMEM, the cultures were fixed with absolute methanol, stained with Giemsa and monitored microscopically (see below). When the effect of LF was to be tested, 0.1 ml of the corresponding LF solution in DMEM+BSA was substituted for the same volume of DMEM+BSA. In all cases, not less than 200 MPM or HBM were screened, recording the number of parasites associated with (i.e., bound to or internalized by) MPM or HBM, the number of MPM or HBM associated with one or more AMA and the number of MPM or HBM not associated with AMA. These values were used to calculate the percentage of MPM associated with AMA and the average number of T. cruzi per 100 MPM or HBM. Each experimental and control condition was tested in triplicate.

Effects of anti-LF IgG and α-methyl mannoside (α-MM). Rabbit anti-LF IgG and α-MM (Sigma), which bind LF, were used in five types of protocols. Protocol No. 1: 0.1 ml anti-LF IgG (final concentration in the culture fluid 3.3 mg IgG/ml) or 0.1 ml α-MM solution (final

concentration in the culture = 0.25M), 0.1 ml LF solution in DMEM+BSA (10 µg/ml), and 0.1 ml of AMA suspension were added in immediate succession to MPM cultures and incubated for 2 hr (5% CO_2). Protocol No. 2: MPM monolayers were incubated with 10 µg LF/ml for 30 min, washed with DMEM and then incubated with anti-LF or α-MM (at the same concentrations as above) for 30 min. After washing with DMEM, the cultures received the AMA and were incubated for 2 hr. Protocol No. 3: MPM monolayers were simultaneously pretreated with 10 µg LF/ml and anti-LF or α-MM (at the same concentrations as above) for 30 min, washed with DMEM and then incubated with the AMA for 2 hr. Protocol No. 4: AMA were preincubated with LF (10 µg/ml) at 37°C for 30 min, washed and then incubated with anti-LF or α-MM for 30 min. After washing with DMEM, these organisms were added to untreated MPM cultures and incubated for 2 hr. Protocol No. 5: AMA were simultaneously preincubated with LF (10 µg/ml) and anti-LF or α-MM at 37°C for 30 min, washed and, after added to untreated MPM cultures, incubated for 2 hr. In all cases, control tests were included in the experiments in which DMEM+BSA was substituted for the tested reagents and normal rabbit IgG was used instead of anti-LF IgG. All conditions were tested in triplicate and the cultures were terminated and processed as described in the preceding paragraph.

Pretreatment of MPM or AMA with LF. To study the effects of pre-incubation of either MPM (5% CO_2 incubator) or AMA (water bath) with LF on their association with the untreated counterpart, monolayers of the former and suspensions of the latter were incubated with 10 µg LF/ml in DMEM+BSA at 37°C for varying periods of time (see Results). After several washings with DMEM, co-cultures of the LF-treated cell with the

untreated counterpart were set up as described above and in the following section. For mock treatments, DMEM+BSA was substituted for the LF solution.

Killing of AMA by MPM or HBM. The co-cultures of MPM or HBM with AMA were set up and incubated for 2 hr as described under Measurement of AMA uptake by MPM. After removing the free AMA, 0.3 ml of fresh DMEM+FBS was added and the MPM monolayers were further incubated for 4 hr. These cultures were terminated and processed for counting as described above. Triplicate assays were set up for each experimental and control condition.

LF treatment of AMA-containing MPM. After incubating MPM cultures with AMA for 2 hr as described under Measurement of AMA uptake by MPM, the free organisms were removed by washing and 0.3 ml of DMEM+BSA alone or containing 10 µg LF/ml was added. These cultures were then incubated for varying periods of time (see Results) and washed with the DMEM. After receiving 0.3 ml DMEM+FBS, the cultures were incubated further until 4 hr had elapsed from the time the free AMA were removed. The cultures were then terminated as described above. In some experiments, culture supernatants were collected at the end of the 4-hr incubation period, centrifuged, decanted and the fluid at the bottom of the tubes was screened microscopically for the presence of free AMA.

Effect of scavengers of oxygen reduction metabolites on AMA killing by MPM. The protocol described under Killing of AMA by MPM was modified so that different scavengers of oxygen reduction intermediates could be incorporated into the system. One tenth of an ml of solutions of recrystalized beef liver catalase (to provide a final concentration in the culture of 160,000 U/ml), SOD (3,000 U/ml), histidine (10 mM) or sodium

benzoate (10 mM) in DMEM+FBS and 0.2 ml DMEM+FBS were added to MPM cultures and incubated for 3 hr.After washing with DMEM, 0.3 ml of LF solution at 10 µg/ml DMEM+BSA (containing the scavenger to be tested at the concentrations mentioned above) was added and the cultures were further incubated for 1 hr. The culture fluids were then removed and replaced with 0.2 ml of AMA suspension plus 0.1 ml of DMEM+BSA containing the corresponding scavenger (to attain the same final concentrations). Cells and parasites were co-cultured for 2-hr. Control cultures were included which were treated with DMEM+BSA instead of LF solution. For heat inactivation, catalase and SOD solutions were heated at 100°C for 20 min. Six identical cultures were set up for each condition; three of these were terminated by washing and fixation (see above) at the end of the 2-hr co-culture period. The remainder were washed and, after receiving 0.3 ml of DMEM+FBS containing the scavenger, were incubated further for 4 hr and then terminated. All cultures were monitored as described above.

Latex bead uptake by LF-treated and mock-treated MPM. Cultures of MPM were either pretreated with LF (10 µg/ml) for 30 min before receiving 0.2 ml of latex bead suspension containing 2.5×10^6 beads/ml (average diameter = 1µm, Sigma) and 0.1 ml DMEM+BSA or received the LF and the latex beads at the same time. Control cultures did not contain LF. The amount of latex beads used in these tests was smaller than usually used in this type of test because we wanted to use as many particles as AMA were used in the AMA uptake assay. After incubating the cultures for 2 hr (5% CO_2), the monolayers were washed, fixed and stained with Giemsa. The percentage of latex-containing MPM and the number of latex beads per 100

MPM were calculated from parameters similar to those measured in MPM-AMA interaction experiments.

Nitroblue tetrazolium test. MPM were incubated with DMEM+BSA alone or containing 10 µg/ LF/ml at 37°C for 1 hr, washed and incubated with AMA in the presence of nitroblue tetrazolium at 0.5 mg/ml for 2 hr. After washing with DMEM, the cultures received 0.3 ml fresh DMEM+FBS and, after another 30 min incubation period, they were fixed with absolute methanol and tested for the presence of insoluble blue/black formazan as described by Murray and Cohn (17).

Immunofluorescence tests for LF binding to AMA, HBM or MPM. AMA, HBM or MPM fixed with 0.25% formaldehyde were incubated (37°C, 30 min) with DMEM+BSA alone or containing 10 or 100 µg LF/ml. After several washings, these preparations were incubated (37°C, 30 min) with heat-inactivated normal rabbit serum, washed and then incubated (37°C, 30 min) with a solution of fluoresceinated anti-LF IgG (10 mg IgG/ml, Cappel). The slides were then washed, air dried and examined with a fluorescence microscope.

^{125}I labeling of LF and determination of receptors for LF on AMA. One half ml LF solution (at 3.1 mg/ml in phosphate-buffered saline solution, pH 7.0, PBS) was mixed with 50 µl of Na^{125}I (specific activity 15 mCi/µg I; Amersham Corp., Arlington Heights, IL) in a vial containing 100 µg Iodogen (Pierce Chemical CO., Rockford, IL) and incubated at room temperature for 15 min. Unbound radioactivity was removed by gel filtration through Sephadex G-25 (Pharmacia, Piscataway, NJ) equilibrated with PBS and the radiolabeled LF was concentrated by ultrafiltration through collodion bags (Schleicher and Schuell, Keene, NH)

The material used in the experiment depicted in Fig. 1 had a specific activity of 8.74×10^5 cpm/µg LF. The number of receptors for LF present on the surface of T. cruzi AMA was determined as follows: in 1.5-ml conical tubes (Bio-Rad, Richmond, CA; catalog 223-9501) pre-coated with a 20% solution of BSA in PBS, 100-µl aliquots of AMA suspension (1.2×10^7 organisms/ml in Hank's balanced salt solution containing 1% BSA) were mixed with an equal volume of PBS containing increasing amounts of ^{125}I-labelled LF. After incubation at 37°C for 1 hr, the non-bound ^{125}I-LF was removed by centrifugation and two washings. Bound ^{125}I-LF was determined by measuring radioactivity in the pellet with a gamma counter and free ^{125}I-LF was calculated by subtracting the amount bound from the total amount added. In competition type of experiments using cold LF the assays were conducted as above except that an additional 100 µl of LF or human hemoglobin (Sigma) solution (both at 3.3 mg/ml in PBS) was added. In these experiments, only one concentration of ^{125}I-LF was used: 250 µg/ml.

Presentation of results and statistical analysis. All results presented in this paper are expressed as mean ± SD. Differences were considered to be statistically significant if $P<0.05$, calculated by the Mann-Whitney "U" test.

RESULTS

Lack of effect of reagents on the viability of AMA, MPM or HBM. None of the reagents used in this work, including LF, affected the viability of MPM or HBM (determined by trypan blue exclusion) when incubated with these cells for up to 9 hr or the viability of T. cruzi AMA (evidenced by their typical vibrational motion in situ when incubated with the parasites for 6 hr under the same conditions as selected for our experiments.

Effects of LF on the interaction of AMA with MPM Presence of LF in co-cultures of MPM or HBM and AMA during the 2-hr incubation period increased cell-parasite association as evidenced by significant elevations in both the percentage of phagocytes associating with AMA and the number of organisms per 100 host cells (Table I). In the two repeat titrations using MPM, these effects were first detectable with either 0.1 or 1 µg LF/ml. However, because the concentration of LF producing a maximal enhancement was 10 µg/ml, it was used in subsequent experiments. At 50 µg LF/ml, there was no significant change in the extent of AMA-MPM association with respect to the control values.

To establish whether LF enhanced AMA-MPM interaction through an effect on the host cell or on the AMA, or on both, experiments were designed in which each of these cells was treated with LF, washed and then co-cultured with the AMA with 10 µg LF/ml for various periods of time significantly increased their association (Table II). The minimal MPM pretreatment time required to significantly increase both the percentage of AMA-associated MPM and the number of AMA per 100 MPM was found to be 30

TABLE I

Enhancing effect of LF on MPM or HBM association with AMA[a]

Cell	LF (μg/ml)	% MPM containing AMA	(%C)[b]	Number of AMA per 100 MPM or HBM	(%C)
MPM	0	12.5 ± 0.7		19.7 ± 0.5	
	0.001	13.6 ± 0.6	(8.8)	20.0 ± 1.4	(1.5)
	0.01	14.5 ± 2.5	(16.0)	19.1 ± 2.5	(-3.0)
	0.1	17.2 ± 1.3[c]	(37.6)	22.3 ± 1.0	(13.2)
	1.0	17.8 ± 1.0[c]	(42.4)	24.6 ± 0.9[c]	(24.9)
	10.0	27.6 ± 4.4[c]	(120.8)	37.6 ± 5.0[c]	(90.9)
	50.0	14.5 ± 1.0	(16.0)	20.8 ± 2.1	(5.6)
HBM	0	21.8 ± 1.6		31.5 ± 0.7	
	10	40.1 ± 1.7[c]	(83.9)	64.1 ± 3.1[c]	(103.5)

For footnotes please see next page

Footnotes to Table I

[a] This set of results is typically representative of two separate experiments with the same protocol. The experiments with MPM and HBM were carried out separately.

[b] %C, percentage of change with respect to the corresponding control value (no LF present).

[c] The difference between this value and its corresponding control value is statistically significant ($P<0.05$).

TABLE II

Effects of pretreatment of MPM or AMA with LF on their capacity to associate with the untreated counterpart[a]

Pretreated cell	Pretreatment	Pretreatment time (min)	% MPM containing AMA (%C)[b]	Number of AMA per 100 MPM (%C)
MPM	DMEM+BSA	10	16.0 ± 1.0	20.0 ± 0.8
MPM	LF[c]	10	16.2 ± 0.6 (1.3)	24.0 ± 1.5 (20.0)
MPM	DMEM+BSA	20	15.2 ± 0.6	19.0 ± 0.7
MPM	LF	20	16.7 ± 0.6 (9.9)	29.5 ± 0.5[d] (55.3)
MPM	DMEM+BSA	30	16.2 ± 0.5	20.5 ± 0.0
MPM	LF	30	20.8 ± 0.7[d] (28.4)	37.3 ± 0.6[d] (81.8)
MPM	DMEM+BSA	60	15.2 ± 0.6	20.7 ± 0.5
MPM	LF	60	22.8 ± 0.6[d] (50.0)	38.3 ± 0.7[d] (85.0)
AMA	DMEM+BSA	10	18.8 ± 1.6	22.3 ± 0.9
AMA	LF	10	23.5 ± 2.5 (25.0)	33.6 ± 3.3[d] (50.7)
AMA	DMEM+BSA	20	16.8 ± 2.3	22.7 ± 2.7
AMA	LF	20	31.5 ± 1.6[d] (87.5)	45.5 ± 2.2[d] (100.4)
AMA	DMEM+BSA	30	20.0 ± 1.3	25.5 ± 3.4
AMA	LF	30	36.8 ± 2.3[d] (84.0)	55.8 ± 2.7[d] (118.8)
AMA	DMEM+BSA	60	16.5 ± 1.0	25.8 ± 1.9
AMA	LF	60	36.8 ± 0.6[d] (123.0)	60.8 ± 2.6[d] (135.7)

For footnotes please see next page.

Footnotes to Table II

[a] This set of results is typically representative of three separate experiments with the same protocol.

[b] %C, percentage of change with respect to the corresponding control value (DMEM+BSA).

[c] In these experiments LF was always used at a final concentration of 10 µg/ml. The experiments in which MPM and AMA were pretreated with LF were carried out separately.

[d] The difference between this value and its corresponding control value is statistically significant ($P<0.05$).

min whereas for the AMA it was 10 min (i.e., when first tested). Next we set out to establish whether HBM, MPM or AMA bound LF. The results of immunofluorescence tests using fluorescein-labeled anti-LF IgG showed that, although untreated HBM, MPM and AMA did not have detectable LF on their surface, approximately 60% of the HBM, 80% of the MPM and 50% of the AMA bound the glycoprotein when the latter was present at 100 µg/ml (Table III). Similar tests performed with 10 µg/LF/ml, i.e., the concentration of LF used in our parasite-host cell interaction assays, detected lower but significant percentages of LF-positive HBM, MPM and similar percentages of LF-positive AMA. The presence of receptors for LF on the surface of macrophages and monocytes has been previously demonstrated (18, 19). The results of experiments in which we measured concentration-dependent binding of ^{125}I-LF to AMA revealed that LF binding reached saturation levels when 96 ng ^{125}I-LF was bound to 1.2 X 10^6 AMA (Fig. 1). Through Scatchard analysis, the data was found to correspond to 1.06 X 10^6 LF receptors per AMA and Ka= 3.0 X 10^5 $l.mol^{-1}$. To test the specificity of the ^{125}I-LF binding to the parasites we carried out competition type of experiments adding unlabeled LF; control experiments were included in which unlabeled hemoglobin was added instead of LF. In the presence of a 13-molar excess of cold LF, a 79% reduction in the uptake of ^{125}I-LF uptake was observed (from 29,199 $\pm$ 2,766 to 6,100 $\pm$ 1,732 cpm) whereas the reduction caused by hemoglobin was insignificant (from 29,199 $\pm$ 2,766 to 27,721 $\pm$ 1,879 cpm).

Inhibition of the LF enhancing effect by agents which bind LF. To establish whether binding of LF to MPM or AMA was required to produce the

TABLE III

Presence of receptors for LF on the surface of MPM, HBM and T. cruzi AMA[a]

Cell	LF pretreatment	LF concentration (µg/ml)	% LF-positive cells
MPM	No	0	0.0 ± 0.0
MPM	Yes	10	19.0 ± 1.0
MPM	Yes	100	83.2 ± 3.0
HBM	No	0	0.0 ± 0.0
HBM	Yes	10	26.1 ± 3.0
HBM	Yes	100	60.0 ± 2.0
AMA	No	0	0.0 ± 0.0
AMA	Yes	10	47.6 ± 2.3
AMA	Yes	100	52.3 ± 2.0

For footnotes please see next page

Footnotes to Table III

[a] This set of results is typically representative of two separate experiments with the same protocol. All of the differences between the experimental values and the corresponding control values are statistically significant ($P<0.05$).

FIGURE LEGEND

Figure 1. Concentration-dependent binding of ^{125}I-LF to T. cruzi amastigotes (left panel) and Scatchard plot analysis of the data (right panel). Data shown are from a representative experiment. The calculated number of LF receptors per organism was 1.1×10^6; $Ka = 3.8 \times 10^5$ $l.mol^{-1}$.

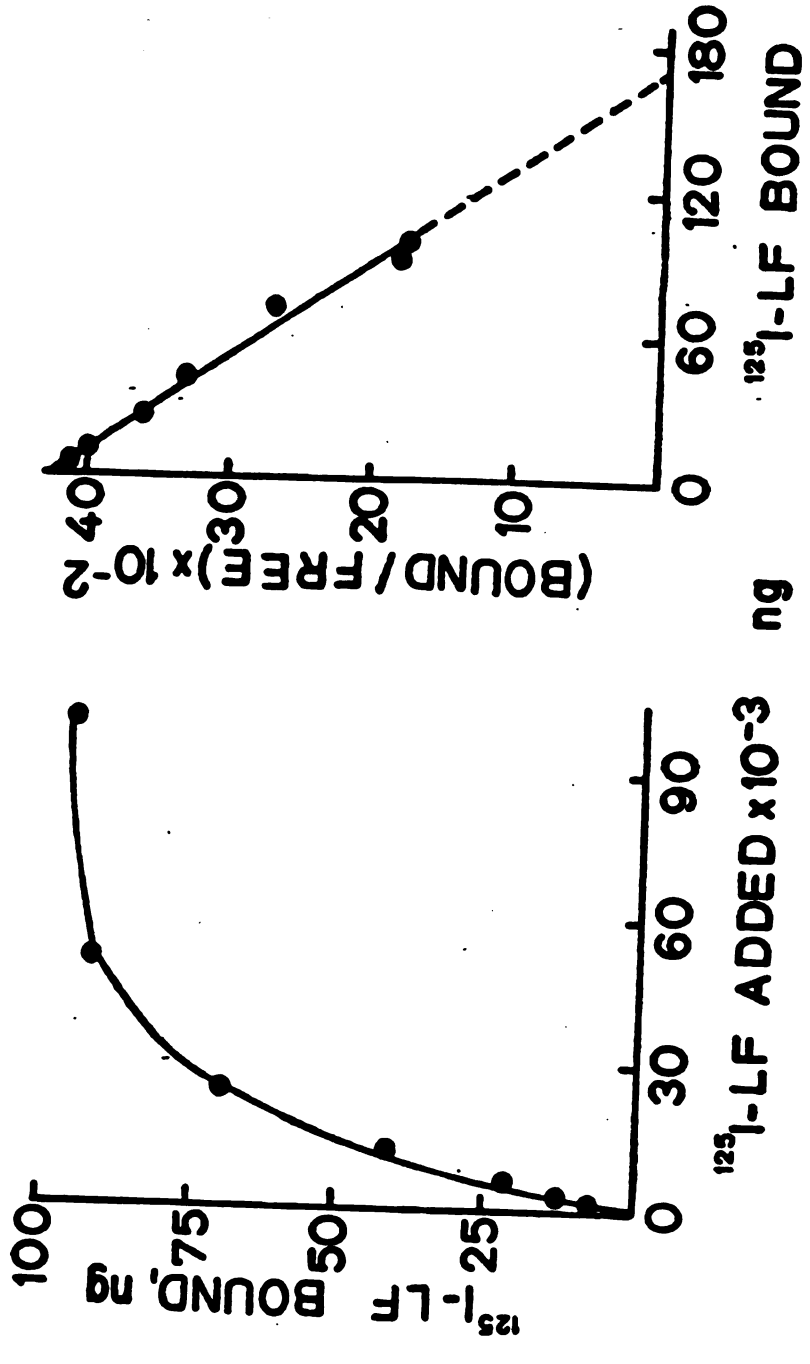
100
75
50
25
0
^{125}I-LF BOUND, ng
0
30
60
90
^{125}I-LF ADDED x10^{-3} ng
(BOUND/FREE)x 10^{-2}
40
30
20
10
0
60
120
180
^{125}I-LF BOUND

Figure 1

enhancing effect, we incorporated into the system an agent known to bind LF. Selected for this purpose were anti-LF IgG and α-MM (18). Five different protocols were designed to clarify not only if LF binding to the interacting cells was a requirement but also if blocking LF bound to MPM or AMA would prevent the enhancing effect (Table IV). As can be seen in Table V, co-cultures of MPM and AMA incubated with LF together with either anti-LF IgG or α-MM (protocol No. 1, see Materials and Methods) resulted in a marked reduction in the extent of the LF enhancing effect. Neither anti-LF nor α-MM affected AMA-macrophage interaction in the absence of LF. Although Table V, shows results obtained with 0.25M α-MM, significant inhibitory effects were also produced by concentrations of this reagent as low as 0.01M (data not shown). When MPM cultures were pretreated with LF and then incubated with the blocking agents before receiving untreated AMA (protocol No. 2), no inhibition of the LF effect was seen. In contrast, when MPM cultures were simultaneously incubated with LF and anti-LF (or α-MM), washed and then co-cultured with untreated AMA (protocol No. 3), the enhancing effect of LF was not seen. The LF effect was markedly inhibited when AMA were pretreated first with LF, then with anti-LF or α-MM and finally incubated with untreated MPM cultures (Table V, protocol No. 4). The LF effect was also abrogated when AMA were incubated with LF in the presence of either anti-LF or α-MM prior to being added to the MPM cultures (protocol No. 5). When normal rabbit IgG was substituted for rabbit anti-LF IgG, the LF effect was not altered significantly in any of the five protocols and normal rabbit IgG had no detectable effect of its own on MPM-AMA association. It is noteworthy that, in all five protocols and in all repeat experiments, the changes in the percentage of MPM

TABLE IV

Summary of protocols 1 through 5

Protocol No. 1

a) [MPM + AMA + LF + anti-LF IgG (or α-MM)] $\xrightarrow{\text{(2 hr at 37°C)}}$

b) terminate by washing and fixation

Protocol No. 2

a) [MPM + LF] $\xrightarrow{\text{(30 min, 37°C)}}$

b) wash, add anti-LF IgG (or α-MM) $\xrightarrow{\text{(30 min, 37°C)}}$

c) wash, add untreated AMA $\xrightarrow{\text{(2 hr at 37°C)}}$

d) terminate by washing and fixation

Protocol No.3

a) [MPM + LF + anti-LF IgG (or α-MM)] $\xrightarrow{\text{(30 min, 37°C)}}$

b) wash, add untreated AMA $\xrightarrow{\text{(2 hr at 37°C)}}$

c) terminate by washing and fixation

Protocol No.4

a) [AMA + LF] $\xrightarrow{\text{(30 min, 37°C)}}$

b) wash, add anti-LF IgG (or α-MM) $\xrightarrow{\text{(30 min, 37°C)}}$

c) wash, add these AMA to untreated MPM cultures $\xrightarrow{\text{(2 hr at 37°C)}}$

d) terminate by washing and fixation

Protocol No.5

a) [AMA + LF + anti-LF IgG (or α-MM)] $\xrightarrow{\text{(30 min, 37°C)}}$

b) wash, add these AMA to untreated MPM cultures $\xrightarrow{\text{(2 hr at 37°C)}}$

c) terminate by washing and fixation

TABLE V

Effects of αc-MM or anti-LF on the enhancing effect produced by pretreatment of MPM with LF or by the presence of LF during MPM-AMA interaction[a]

Reagents tested[b]	Number of AMA per 100 MPM measured in		
	Protocol No. 1 (%C)[c]	Protocol No. 2 (%C)	Protocol No. 3 (%C)
DMEM+BSA	37.5 ± 1.7	17.5 ± 0.5	20.2 ± 3.3
LF	65.3 ± 4.3[d] (74.1)	38.7 ± 1.6[d] (121.1)	33.5 ± 2.0[d] (65.8)
LF, αc-MM	36.2 ± 1.6 (-3.5)	33.8 ± 1.0[d] (93.1)	22.0 ± 1.9 (8.9)
αc-MM	37.3 ± 2.3 (-0.5)	18.3 ± 1.3 (4.6)	21.0 ± 1.4 (4.0)
DMEM+BSA	21.8 ± 0.9	17.5 ± 0.5	20.2 ± 3.3
LF	37.1 ± 2.0[d] (70.2)	38.7 ± 1.6[d] (121.1)	33.5 ± 2.0[d] (65.8)
LF, anti-LF	18.0 ± 0.9 (-17.4)	33.5 ± 2.7[d] (91.4)	22.3 ± 2.2 (10.4)
Anti-LF	19.0 ± 1.4 (-12.8)	19.0 ± 1.3 (8.6)	22.2 ± 1.9 (9.9)
LF, normal rabbit IgG	35.0 ± 1.5[d] (60.6)	35.2 ± 1.7[d] (101.1)	38.0 ± 1.9[d] (88.1)
Normal rabbit IgG	20.0 ± 1.2 (-8.3)	19.5 ± 0.4 (11.4)	21.0 ± 1.4 (4.0)

For footnotes please see next page

Footnotes to Table V

[a] The sets of results for each protocol are typically representative of two to three separate experiments with the same protocol. For protocol No. 1, the experiments with α_2-MM and anti-LF were carried out separately. For the description of protocols No. 1, 2 and 3 see Materials and Methods.

[b] The final concentrations of LF, α_2-MM, anti-LF IgG and normal rabbit IgG used in these experiments were 10 µg/ml, 0.25M, 3.3 mg/ml and 3.3 mg/ml, respectively.

[c] See footnote b under Table II.

[d] The difference between this value and the corresponding control (DMEM+BSA) was statistically significant ($P<0.05$).

containing AMA were of the same direction as those shown in Table V and VI for the number of AMA per 100 MPM (data not shown).

Effects of LF on latex bead uptake by MPM. To determine if the enhancing effect of LF on MPM was unique for AMA, we performed identical experiments using latex beads. The results indicated that the presence of LF during incubation of MPM with the beads or LF treatment of the MPM prior to adding the particles significantly enhanced particle uptake (Table VII).

Effects of LF on AMA killing by HBM or MPM. While the results presented above demonstrate the increased capacity of LF-treated MPM to take up AMA and latex particles, they did not reveal whether LF-treated MPM would also display increased killing capacity. To test this possibility, we set up experiments with HBM or MPM in which 10 μg LF/ml was present during the 2-hr cell-parasite interaction period and monitored the decrease in parasite load 4 hr after removing both LF and the non-bound AMA. Selection of the 4-hr incubation period was based on previous results revealing that untreated MPM destroyed a small number of AMA 6 hr after initiation of the MPM-AMA interaction (2). The results presented in Table VIII showed that HBM and MPM destroyed significantly larger numbers of AMA after exposed to LF. In separate experiments, MPM that had been pretreated with LF for 60 min and then incubated with untreated AMA for 2 hr cleared a larger number of parasites than mock-treated MPM over the 4-hr period (Table IX). At this point, we were curious to find out whether pretreatment of the AMA with LF would also lead to their more rapid destruction by untreated MPM. The results presented in Table VIII showed that this was indeed the case. Of

TABLE VI

Inhibition by α-MM or anti-LF of the enhancing effect of LF pretreatment of AMA on MPM-AMA association[a]

Reagents tested[b]	Number of AMA per 100 MPM measured in	
	Protocol No. 4 (%C)[c]	Protocol No. 5 (%C)
DMEM+BSA	25.8 ± 2.3	18.7 ± 2.0
LF	55.6 ± 1.7[d] (115.5)	34.2 ± 2.4[d] (82.9)
LF, α-MM	27.2 ± 2.1 (5.4)	20.5 ± 0.4 (9.6)
α-MM	27.8 ± 1.8 (7.8)	19.3 ± 2.4 (3.2)
LF, anti-LF	30.0 ± 0.8 (16.3)	18.3 ± 2.0 (-2.1)
Anti-LF	28.8 ± 2.4 (11.6)	20.5 ± 1.4 (9.6)
LF, normal rabbit IgG	49.7 ± 5.2[d] (92.6)	33.7 ± 0.9[d] (80.2)
Normal rabbit IgG	32.5 ± 0.8 (26.0)	17.7 ± 1.0 (-5.3)

For footnotes please see next page

Footnotes to Table VI

[a] The sets of results for each protocol are typically representative of two to three separate experiments with the same protocol. For the results obtained when LF was present during AMA-MPM interaction, see Table IV, protocol No. 1. For the description of protocols 4 and 5 see Materials and Methods.

[b,c,d] See footnotes b,c,d under Table IV.

TABLE VII

Effect of LF on the capacity of MPM to take up latex beads[a]

Exp. No.	MPM treatment[b] Step A	Step B	Number of latex beads per 100 MPM (%C)[c]
1	DMEM+BSA	DMEM+BSA	44.0 ± 3.7
	DMEM+BSA	LF (10 µg/ml)	85.3 ± 5.0[d] (93.9)
2	DMEM+BSA	DMEM+BSA	45.3 ± 3.3
	LF (10 µg/ml)	DMEM+BSA	71.3 ± 2.1[d] (57.4)

For footnotes please see next page

Footnotes to Table VII

[a] Each set of results is typically representative of two separate experiments with the same protocol.

[b] Step A consisted of incubating MPM cultures with the indicated solution at 37°C for 30 min and was immediately followed by step B, which consisted of incubating these MPM with the indicated solution plus latex beads for 2 hr.

[c] See footnote b under Table II.

[d] The difference between this value and the corresponding control (DMEM+BSA) is statistically significant ($P<0.05$).

TABLE VIII

Effects of the presence of LF during co-culture of HBM or MPM with AMA on their capacity to kill the parasite[a]

Cell	LF treatment[b]	Number of AMA per 100 MPM after			% AMA killed[c]
		2 hr[d]	(%C)[e]	6 hr[d]	
HBM	No	23.0 ± 2.8		21.0 ± 2.1	8.7
HBM	Yes	38.6 ± 2.7[f]	(67.8)	9.6 ± 1.6	75.1[g]
MPM	No	16.7 ± 1.8		14.3 ± 1.3	14.4
MPM	Yes	35.0 ± 2.6[f]	(84.0)	8.0 ± 1.5	77.1[g]

For footnotes please see next page

Footnotes to Table VIII

[a] This set of results is typically representative of two separate experiments with the same protocol.

[b] DMEM+BSA alone or containing 10 µg LF/ml. LF was present during the 2-hr co-culture of phagocytic cells with AMA.

[c] The percentage of AMA killed during the 4-hr period was calculated by the equation:

$$\% \text{ AMA killed} = \frac{\text{2-hr value - 6-hr value}}{\text{2-hr value}} \times 100.$$

[d] Values given under the 2- and 6-hr columns were measured in cultures terminated immediately after removal of the free AMA (i.e., after 2 hr of AMA-MPM interaction) and 4 hr later, respectively.

[e] See footnote b under Table II.

[f] The difference between this value and the corresponding control was statistically significant ($P<0.05$).

[g] This extent of killing was statistically different from that seen with cells mock-treated with DMEM+BSA ($P<0.05$).

TABLE IX

Effects of pretreatment of MPM or AMA with LF on their capacity to kill AMA[a]

Pretreated cell	Pretreatment[b]	Number of AMA per 100 MPM after 2 hr[d] (%C)[e]	Number of AMA per 100 MPM after 6 hr[d]	% AMA killed[c]
MPM	DMEM+BSA	20.7 ± 0.5	17.3 ± 0.8	16.4
MPM	LF (10 µg/ml)	38.3 ± 0.7[f] (85.0)	9.3 ± 0.1	75.7[g]
AMA	DMEM+BSA	25.8 ± 1.9	23.8 ± 1.9	7.8
AMA	LF (10 µg/ml)	60.8 ± 2.6[f] (135.7)	18.5 ± 1.5	69.6[g]

[a,c,d,e,f,g] See footnotes under Table VII.

[b] Sixty-minute incubation with the indicated solution.

particular interest was the observation that untreated MPM that had taken up untreated AMA also killed the organisms at a faster rate if LF was added to the culture (Table X). It is noteworthy that, in all of the experiments designed to monitor AMA killing, free AMA were virtually undetectable in the culture medium at the end of the experiment, rendering unlikely the possibility that reductions in parasite load could have resulted from detachment of surface-bound organisms.

Mechanism of killing of AMA by LF-treated MPM. A recent report from our laboratory has documented that the killing of T. cruzi AMA by MPM involves H_2O_2 (2). To test whether LF-treated MPM killed the parasites by a similar mechanism, we set up experiments using scavengers of oxygen reduction intermediates. Catalase (scavenger of H_2O_2) did not affect the enhancing effect of LF on MPM-AMA association but completely abrogated parasite destruction (Table XI). Heat-inactivated (100°C, 20 min) catalase did not inhibit killing. Histidine (scavenger of $^1O_2$) and SOD (scavenger of $O_2^{\cdot-}$) had no effect on AMA uptake by LF-treated MPM but partially inhibited killing. Heat-inactivated SOD had no effect on either parameter. That $O_2^{\cdot-}$ was indeed produced by MPM that had internalized AMA was confirmed by a) the observation that, among the AMA-containing MPM, 83% of the cells gave a positive nitroblue tetrazolium reduction test whereas only 44% were positive if mock-treated with medium and b) the finding that only 15% and 12% of these cells, respectively, gave a positive nitroblue tetrazolium test if SOD was present before and during cell-parasite interaction.

TABLE X

Effect of addition of LF to cultures of MPM containing AMA on parasite killing[a]

Solution added after removal of free AMA	Length of treatment[c] (hr)	Number of AMA per 100 MPM	% AMA killed[b]
None[d]		27.8 ± 4.7	
DMEM+BSA	1	24.3 ± 3.8	12.6
LF	1	11.8 ± 0.9	57.6[e]
DMEM+BSA	2	24.3 ± 3.0	12.6
LF	2	11.6 ± 0.2	58.3[e]
DMEM+BSA	3	24.8 ± 0.6	10.8
LF	3	12.3 ± 0.6	55.8[e]

For footnotes please see next page

Footnotes to Table X

[a] This set of results is typically representative of two separate experiments with the same protocol.

[b] Percentage of reduction in parasite load during the 4-hr period (reference value 27.8).

[c] LF (10 µg/ml) or DMEM+BSA was added immediately after removal of the free AMA and left in the cultures for the indicated amounts of time. After removing the LF by washing, the cultures were incubated with fresh DMEM+FBS for the remainder of the 4-hr period.

[d] The value shown in this line was obtained immediately after removal of the free AMA, i.e., after 2 hr of co-culture of MPM and AMA. All other values in this table were obtained 4 hr later.

[e] This extent of killing was statistically different from that produced by cells mock-treated with DMEM+BSA ($P<0.05$).

TABLE XI

Effects of scavengers of intermediates of oxygen reduction on the LF-induced increase of AMA killing by MPM[a]

Exp. No.	Reagent or condition	Number of AMA per 100 MPM measured after 2 hr	Number of AMA per 100 MPM measured after 6 hr	% AMA killed[b]
1	DMEM+BSA	16.7 ± 0.6	16.0 ± 0.9	4.2
	LF	49.0 ± 2.3[c]	13.8 ± 0.9	71.8[d]
	Catalase + LF	49.5 ± 0.4[c]	49.8 ± 3.9	-0.6
	Catalase	17.2 ± 1.7	16.8 ± 0.9	2.3
	Heated catalase + LF	45.0 ± 2.9[c]	12.5 ± 1.3	72.2[d]
	SOD + LF	50.7 ± 2.3[c]	32.8 ± 4.4	35.3[d]
	SOD	17.2 ± 1.0	15.3 ± 1.3	11.0
	Heated SOD + LF	47.5 ± 3.5[c]	12.0 ± 1.4	74.7
	Heated SOD	16.6 ± 1.7	16.8 ± 1.3	-1.2
2	DMEM + BSA	28.0 ± 3.8	27.8 ± 4.9	0.7
	LF	62.7 ± 2.9[c]	18.3 ± 1.3	70.8[d]
	Sodium benzoate + LF	49.8 ± 4.0[c]	18.2 ± 4.3	63.5[d,e]
	Sodium benzoate	27.5 ± 1.8	25.5 ± 3.0	7.3
	Histidine + LF	58.0 ± 2.6[c]	39.8 ± 3.7	31.4[d]
	Histidine	27.0 ± 2.7	28.2 ± 3.0	-4.4

Footnotes to Table XI:

[a] This set of results is typically representative of two separate experiments with the same protocol.

[b] This percentage represents the reduction in parasite load during the 4-hr period.

[c] The difference between this value and the corresponding control (DMEM+BSA) is statistically significant ($P<0.05$). In each experiment, all LF effects (2-hr values) are statistically comparable.

[d] The difference between this extent of killing and that seen in the control (DMEM+BSA) is statistically significant ($P<0.05$).

[e] The difference between this extent of killing and that seen with LF alone is not statistically significant ($P<0.05$).

DISCUSSION

These results show that LF increases the uptake and intracellular destruction of intracellular forms of a protozoan pathogen by HBM or MPM. Concentrations of LF enhancing the interaction of the phagocytic cells with T. cruzi AMA ranged from 0.1 to 10 µg/ml. These levels compare with normal human plasma concentrations of 1.5 ±1.8 µg/ml (20), and pathological levels varying between 4 and 28 µg/ml in cases of burn injury (21), 12 to 22 µg/ml in cases of chronic myeloid leukemia in relapse and 5 and 12 µg/ml in two cases of hypersplenism secondary to hepatic cirrhosis (20). At higher concentrations -50 µg/ml- LF did not produce an enhancing effect (Table I) and we thought this might owe to saturation of LF receptors on the surface of MPM and AMA and that at lower, effective concentrations, LF might bridge the interacting cells. For this hypothesis to be valid two conditions had to be met: i) LF would have to bind to the surface of both the AMA and the phagocytes and ii) pretreatment of either MPM or AMA would have to increase association of the treated cell with the untreated counterpart. The results of our immunofluorescence studies showed that HBM, MPM and AMA indeed could bind LF. Of note, the results obtained with HBM and MPM are in agreement with those of other investigators who reported that radiolabeled LF binds to HBM as well as to MPM (15, 19, 22-23). The results of the experiments in which we measured concentration-dependent binding of ^{125}I-LF to AMA and determined the ability of cold LF to compete with radiolabeled LF for binding sites on the parasites indicated that LF bound specific receptors on the AMA surface. As to the second condition, the pretreatment of

either AMA or MPM with LF did result in significantly enhanced association of these cells with the untreated counterpart. While these results appeared to support the hypothesis that LF bridged phagocytes and AMA, additional experiments were carried out to confirm it. If LF increased the association merely by bridging MPM and AMA, blocking agents such as anti-LF and α-MM would be expected to inhibit at least in part the effect both when present during LF incubation with either cell and when used to treat MPM or AMA that had already been treated with LF (i.e., immediately before being exposed to the untreated counterpart). The results showed that, whenever the experimental conditions allowed the blocking of LF by α-MM or anti-LF IgG in the fluid phase (i.e., during cell treatment; protocols No. 1, 3 and 5), AMA-MPM association was indeed inhibited (Tables V and VI). However, when MPM were pretreated with LF, washed, and then incubated with either blocking agent (protocol No. 2), the LF effect was not significantly inhibited. This observation implied that LF binding to the MPM surface was necessary for this cell to display the enhancing effect and that subsequent treatment with either blocking agent could not inhibit it or reverse it. Because bridging in protocol No. 2 should have been substantially prevented by anti-LF or α-MM but was not, we surmised that bridging was probably not the major factor underlying the LF effect.

In comparing the results obtained with all five protocols we noticed that the LF effect had been seen every time that the MPM had been directly exposed to LF either present in the fluid phase (even if blocked afterwards) or unhindered on the AMA surface. The notion that LF contact with the MPM was required to produce the enhancing effect was supported by the results of protocol No. 4, showing that blocking LF bound to AMA prior

to addition to the MPM cultures abrogated the LF effect (Table VI). Hence, we formulated a new hypothesis, that LF activated MPM, and proceeded to test it. First, we observed that MPM treated with LF either prior to or during incubation with latex beads took up significantly greater numbers of particles than did mock-treated MPM (Table VII). There was also an increase in the proportion of MPM taking up latex beads.

An independent indication of HBM or MPM activation by LF was provided by the results of the experiments in which parasite killing was monitored, particularly under a condition (6-hr cell-parasite interaction period) allowing minimal AMA killing by untreated HBM or MPM (2). Both HBM and MPM disposed of relatively large numbers of AMA, significantly greater than those killed by mock-treated cells, regardless of whether LF was present during the initial 2-hr cell-parasite interaction (Table VIII) or used only to treat MPM or AMA prior to their interaction with the untreated counterpart (Table IX). The large, significant difference between the extents of AMA killed by HBM or MPM exposed to LF (>75%) and by mock-treated phagocytes (<16.4%) denoted the ability of LF to increase the cytolytic capacities of HBM and MPM. A similar increase in cytolysis was seen when untreated MPM were exposed to AMA which had been treated with LF in an otherwise identical experiment (Table IX). Consequently, the MPM appeared to mount the LF effects (i.e., for uptake and killing) regardless of whether the glycoprotein was presented to them in the fluid phase or bound to the AMA surface.

If indeed LF activates MPM, untreated MPM which have already engulfed AMA would be expected to display an increased killing capacity after treated with the glycoprotein. This was in fact the case as

evidenced by the greater percentages of parasites killed by AMA-containing MPM when they were treated with LF (Table X). These results, together with those of Ambruso and Johnston (24), who postulated that LF can activate neutrophils, make it tempting to speculate that LF may be a common activator of different types of inflammatory cells.

Although activation of MPM by LF might by itself account for the entire LF effect, a possible contributory role of LF bridging can not be totally ruled out and the lack of effect of 50 µg LF/ml (Table I) remains to be explained. Conceivably, this high concentration could be supraoptimal, reminiscent of the lack of effect of excess doses of concanavalin A on lymphocyte proliferative responses.

The results of experiments in which scavengers of intermediates of oxygen reduction were present in cultures before, during and after MPM interaction with AMA indicated that H_2O_2 was the chemical species mediating most of the parasite killing, although $O_2^{\cdot-}$ and $^1O_2$ also played a role (Table XI). Previous studies with unelicited MPM showed that these cells could kill T. cruzi AMA via H_2O_2 and that neither SOD (scavenger of $O_2^{\cdot-}$) nor histidine (scavenger of $^1O_2$) had significant inhibitory effects (2). The present results, showing that the latter two oxygen metabolites were also involved in AMA killing by LF-treated MPM, suggest that additional cytolytic resources available to MPM could be recruited to the action upon LF stimulation. Production of $O_2^{\cdot-}$ by LF-treated MPM that had internalized AMA was also evidenced by the reduction of nitroblue tetrazolium to formazan; the percentage of cells containing AMA and formazan among LF-treated MPM was much greater than among mock treated MPM. Interestingly, LF has been shown to induce

production of $OH^{\cdot}$ in human neutrophils (24).

LF shares with fibronectin (8), another glycoprotein produced by an inflammatory cell, an ability to enhance macrophage interaction with T. cruzi. Conceivably, other proteins of inflammatory cell origin may play a role in the uptake and clearance of T. cruzi and perhaps other microorganisms as well, contributing to host resistance mechanisms against infections.

REFERENCES

1. Carvalho, R. M., M. N. L.Meirelles, W. de Sousa, and W. Leon. 1981. Isolation of the intracellular stage of Trypanosoma cruzi and its interaction with mouse macrophages in. vitro. Infect. Immun.33:546.
2. Villalta, F., and F. Kierszenbaum. 1984. Role of inflammatory cells in Chagas' disease. II. Interactions of mouse macrophages and human monocytes with intracellular forms of Trypanosoma cruzi: uptake and mechanism of destruction. J. Immunol. 133:3338.
3. Kierszenbaum, F., E. Knecht, D. B. Budzko, and M. C. Pizzimenti. 1974. Phagocytosis: a defense mechanism against infection with Trypanosoma cruzi. J. Immunol. 112:1839.
4. Williams, D. M., and J. S. Remington. 1977. Effects of human monocytes and macrophages on Trypanosoma cruzi.Immunology 32:19.
5. Villalta, F., and F. Kierszenbaum. 1983. Role of polymorphonuclear cells in Chagas' disease. I. Uptake and mechanisms of destruction of intracellular (amastigote) forms of Trypanosoma cruzi by human neutrophils. J. Immunol. 131:1504.
6. Villalta, F., and F. Kierszenbaum. 1984. Role of inflammatory cells in Chagas' disease. I. Uptake and mechanisms of destruction of intracellular (amastigote) forms of Trypanosoma cruzi by human eosinophils. J. Immunol. 132:2953.
7. Mosesson, M. W., and D. L. Amrani. 1980. The structure and biologic activities of plasma fibronectins. Blood 56:145.

8. Wirth, J. J., and F. Kierszenbaum. 1984. Fibronectin enhances macrophage association with invasive forms of Trypanosoma cruzi. J. Immunol. 133:460.

9. Romaña, C. 1963. Enfermedad de Chagas. Lopez Libreros Editores, Buenos Aires, P. 97.

10. Spitznagel, J. K. 1984. Nonoxidative antimicrobial reactions of leukocytes. In Regulation of Leukocyte Function. Edited by R. Snyderman. Plenum Publishing Corp., New York. P. 283.

11. Laffrell, M. S., and J. K. Spitznagel. 1974. Intracellular and extracellular degranulation of human polymorphonuclear azurophil and specific granules induced by immune complexes. Infect.Immun. 10:1241.

12. Pryzwansky, K. B., E. K. Macrae, J. K. Spitznagel, and M. H. Cooney. 1979. Early degranulation of human neutrophils: immunocytochemical studies of surface and intracellular phagocytic events. Cell 18:1025.

13. Hansen, N. E., H. Karle, V. Andersen, J. Malmquist, and G. E. Hoff. 1976. Neutrophilic granulocytes in acute bacterial infection. Sequential studies on lysozyme, myeloperoxidase and lactoferrin. Clin. Exp.Immunol. 26:463.

14. Nishiya, K., and D. A. Horwitz. 1982. Contrasting effects of lactoferrin on human lymphocyte and monocyte natural killer activity and antibody-dependent cell-mediated cytotoxicity. J. Immunol. 129:2519.

15. Villalta, F., and F. Kierszenbaum. 1982. Growth of isolated amastigotes of Trypanosoma cruzi in cell-free medium. J.Protozool. 29:570.

16. Villalta, F., and F. Kierszenbaum. 1983. Role of cell surface mannose residues in host cell invasion by Trypanosoma cruzi. Biochim. Biophys. Acta 736:39.

17. Murray, H. W., and Z. A. Cohn. 1980. Macrophage oxygen-dependent antimicrobial activity. III. Enhanced oxidative metabolism as an expression of macrophage activation. J. Exp. Med. 152:1596.

18. Bennett, R. M., and J. Davis. 1981. Lactoferrin binding to human peripheral blood cells: an interaction with a β-enriched population of lymphocytes and a subpopulation of adherent mononuclear cells. J.Immunol. 127:1211.

19. Van Snick, J. R., and P. L. Masson. 1976. The binding of human lactoferrin to mouse peritoneal cells. J. Exp. Med. 144:1568.

20. Bennett, R. M., and C. Mohla. 1976. A solid phase radioimmunoassay for the measurement of lactoferrin in human plasma and disease. J. Lab. Clin. Med. 88:156.

21. Wolach, B., T. D. Coates, T. E. Hugli, R. L. Baehner and L. A. Boxer. 1984. Plasma lactoferrin reflects granulocyte activation in burn patients. J. Lab. Clin. Med. 103:284.

22. Van Snick, J. L., P. L. Masson, and J. F. Heremans. 1974. The involvement of lactoferrin in the hyposideremia of acute inflammation. J. Exp. Med. 140:168.

23. Imber, M. J., S. V. Pizzo, W. J. Johnson, and D. O. Adams. 1982. Selective diminution of the binding of mannose by murine macrophages in the late stages of activation. J. Biol. Chem. 257:5129.

24. Ambruso, D. R., and R. B. Jonhston. 1981. Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system. J. Clin Invest. 67:352.

CHAPTER II

LACTOFERRIN EFFECTS ON THE INTERACTION OF BLOOD FORMS OF *Trypanosoma cruzi* WITH MONONUCLEAR PHAGOCYTES

ABSTRACT

Pretreatment of mouse peritoneal macrophages (MPM) or human blood monocytes (HBM) with lactoferrin (LF) -a glycoprotein secreted by neutrophils, whose levels increase in inflammatory conditions- modified the outcome of the interaction between these cells and blood (trypomastigote) forms of Trypanosoma cruzi. A 24-hr LF pretreatment was required for MPM to display an increased capacity to take up the organisms whereas HBM -whose density of surface LF receptors is greater than that of MPM- required a pretreatment of only 2 hr. Since the extent of trypomastigote incorporation by untreated MPM was not significantly affected by treating the former with LF, and indirect immunofluorescence tests failed to reveal the presence of LF receptors on the surface of these organisms, it appeared that the effect of LF was mostly on MPM. When MPM were treated with LF for 2 hr and then exposed to the trypomastigotes for 2 hr, the numbers of parasites per 100 MPM recorded 70 hr later were smaller than those of mock-treated MPM, but there was no significant difference in the proportions of MPM taking up parasites. MPM which had ingested trypomastigotes and were then treated with LF also cleared the organisms faster than mock-treated MPM. Lactoferrin pretreatment also increased the trypanocidal capacity of HBM. H_2O_2 was involved in parasite killing by LF-treated MPM since catalase prevented it. These results show that a neutrophil product can facilitate the uptake and disposal of blood forms of T. cruzi by mononuclear phagocytes and suggest that LF may influence host defense mechanisms against infection with this parasite.

INTRODUCTION

Although inflammatory cell infiltration is one of the early developments in the pathology of Chagas' disease (reviewed by Andrade & Andrade, 1979 and by Romaña, 1963), its precise role in host defense or pathogenesis remains to be defined. Several investigators have examined interactions between some types of inflammatory cells and mammalian forms of Trypanosoma cruzi. This yielded information about the ability of macrophages or monocytes to take up and destroy bloodstream (trypomastigote)(Alcantara & Brener, 1980; De Almeida Maria, Alcantara & Brener, 1982; Kierszenbaum, Knecht, Budzko & Pizzimenti, 1974; Nathan, Nogueira, Juangbhanich, Ellis & Cohn, 1979; reviewed by Scott & Snary, 1982) and intracellular (amastigote) forms of the parasite (Carvalho, Meirelles, De Souza & Leon, 1981; Villalta & Kierszenbaum, 1984, 1984a), and the capacity of eosinophils and neutrophils to destroy these organisms via antibody-dependent cell-mediated cytotoxicity (Kierszenbaum, 1979; Kierszenbaum & Hayes, 1980; Okabe, Kipnis, Calich & Dias da Silva, 1980) or after phagocytosis (Kierszenbaum et al., 1974; Kierszenbaum, Villalta & Tai, 1986). There have also been reports concerning biochemical factors and reactions involved in the uptake of blood forms of T. cruzi by macrophages (Connelly & Kierszenbaum, 1984, 1985; Villalta & Kierszenbaum, 1983, 1983a; Wirth & Kierszenbaum, 1982, 1983). Some of our recent efforts have focused on proteins associated with inflammatory conditions and their ability to modulate the outcome of the interaction of the two mammalian forms of T. cruzi with inflammatory-type cells (Wirth & Kierszenbaum, 1984; Villalta & Kierszenbaum, 1986). We first examined the

effects of lactoferrin (LF) -an iron-binding glycoprotein produced by neutrophils in increased amounts during inflammatory conditions (Pryswansky, Macrae, Spitznagel & Cooney, 1979)- on the fate of amastigotes in mononuclear phagocytic cells (Lima & Kierszenbaum, 1985). In this paper we report the effects of LF on mononuclear phagocyte interaction with the trypomastigote form.

MATERIALS AND METHODS

Animals. Four to six-week-old Crl:CD-1(ICR)BR Swiss mice were purchased from Charles River Laboratory (Portage, Michigan).

T. cruzi. Tulahuén strain trypomastigotes were isolated from blood of mice infected intraperitoneally 2 weeks previously with 2×10^5 trypomastigotes. The organisms were separated from the blood cells by density gradient centrifugation over Isolymph (Gallard Schlesinger, Carle Place, New York) (Budzko & Kierszenbaum, 1974) followed by chromatography through a diethylaminoethyl-cellulose column (Villalta & Leon, 1979). The flagellates were washed with Dulbecco's modified minimal essential medium containing penicillin (100 units/ml) and streptomycin (100 µg/ml) (DMEM) and resuspended at 1×10^7 organisms/ml in the same medium supplemented with either 1% bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri) (DMEM+BSA) or 10% fetal bovine serum (Gibco, Grand Island, New York) (DMEM+FBS). Parasite viability was always >99.8%.

Mouse peritoneal macrophages (MPM). Unstimulated mice were sacrificed by excess ether inhalation. The methods for collecting the resident peritoneal macrophages and for setting up monolayers of these cells on Lab-Tek microscope slide tissue culture chambers have been described in detail elsewhere (Zenian & Kierszenbaum, 1982). These monolayers were incubated at 37°C for 18 hr in a 5% CO_2-in-air incubator (saturated with water vapor) and washed immediately before being subjected to the appropriate treatment. These cultures consisted of >98% nonspecific-esterase-positive cells with typical macrophage morphology.

Human blood monocytes (HBM). Blood was drawn from healthy donors. The

methods to purify monocytes and to prepare adherent monolayers on Lab-Tek tissue culture chambers have been described (Villalta & Kierszenbaum, 1984a). These monolayers were incubated at 37°C for 2 hr in a 5% CO_2 incubator and washed with DMEM prior to further treatments. These cultures consisted of>99% nonspecific-esterase-positive, trypan-blue-excluding monocytes.

Pretreatment of MPM or HBM with LF. Monolayers of MPM or HBM were incubated at 37°C for variable periods of time with 0.3 ml of DMEM+FBS alone or containing varying concentrations of LF (see Results). Unless otherwise stated, LF was removed from the cultures by three washings with DMEM prior to incorporating T. cruzi into the cultures (see below). The batches of human LF (at 10% iron saturation) were purchased from Sigma.

Pretreatment of trypomastigotes with LF. Nine volumes of parasite suspension were mixed with one volume of DMEM+BSA alone or containing LF solution at ten times the desired final concentration in the reaction mixtures and incubated at 37°C for 2 hr . After washing the trypanosomes twice with DMEM by centrifugation (800 X G, 4°C, 20 min), they were resuspended in DMEM+BSA at 1 X 10^7organisms /ml.

Determination of T. cruzi uptake by MPM or HBM. All experimental and control conditions were tested in triplicate. Lactoferrin-treated or mock-treated MPM or HBM monolayers received 0.3 ml of the appropriate parasite suspension and incubated at 37°C and 5% CO_2 for 2 hr . After removing the free parasites by three washings with DMEM, the cultures were fixed with absolute methanol and stained with Giemsa. Not less than 200 cells were screened microscopically (X1000), recording the total number of screened cells, the number of parasites associated with the screened cells

and the number of MPM or HBM associated with one or more trypomastigotes. These values were used to calculate the percentage of MPM or HBM with parasites and the average number of organisms per 100 cells.

Determination of parasite killing by MPM or HBM. Co-cultures of T. cruzi and MPM or HBM were incubated at 37°C and 5% CO_2 for 2 hr as described in the preceding subsection. After removing the parasites, 0.3 ml of fresh DMEM+FBS was added and the cell monolayers were further incubated under the same conditions for various periods of time. These cultures were terminated and processed for counting as described above. All tests were performed in triplicate. In some experiments, untreated MPM which had already internalized untreated parasites were tested for their capacity to kill the latter after LF was added to the culture medium. In this case, MPM monolayers were co-cultured with the parasites as described above and, after removing the free flagellates, 0.3 ml of DMEM+FBS alone or containing 10 µg LF/ml was added. Replicates of these cultures were then incubated at 37°C for various periods of time, washed with DMEM and terminated by fixation as described above. In some experiments, catalase (recrystallized beef liver catalase, Sigma) was added in 0.4 ml DMEM+FBS to attain a final concentration in the cultures of 160,000 units/ml and remained present in the culture medium throughout the incubation period. Heat-inactivated (100°C, 20 min) catalase was used in control assays.

Immunofluorescence assay for LF. Binding of LF by T. cruzi trypomastigotes was tested by incubating parasites fixed with 0.25% formaldehyde in phosphate-buffered saline pH 7.0 (PBS) smeared on microscope slides with solutions containing 10 or 100 µg LF/ml in

DMEM+BSA for 2 hr . After washing three times with PBS, the smears were incubated with fluorescein-labeled rabbit anti-human LF IgG (Cappel Laboratories, West Chester, Pennsylvania) at 37°C for 30 min, washed again with PBS, air dried, and examined by fluorescence microscopy.

Presentation of results and statistics. All results are expressed as the mean $\pm$ 1 S.D. Differences were considered to be significant if $P \leq 0.05$ as determined by Student's "t" test. The sets of results presented in the tables are typically representative of two to four repeat experiments.

RESULTS

Effects of LF on the uptake of *T. cruzi* trypomastigotes by MPM or HBM. Pretreatent of MPM with 10 µg LF/ml altered their capacity to take up blood forms of *T. cruzi* as revealed by increases in both the percentage of parasite-containing MPM and the average number of organisms per 100 MPM; these increases were seen immediately after removing the non-bound parasites (i.e., at 0 hr) (Table 1). Although this effect was consistently produced by a 24-hr pretreatment of the MPM with LF, a relatively small but nevertheless significant increase was seen in some experiments (e.g., the one represented in Table 1) after a 12-hr pretreatment. Experiments performed with HBM showed that these cells were also capable of greater parasite uptake when pretreated with LF but, in this case, the effect was seen after pretreating the HBM with either 10 or 100 µg LF/ml for only 2 hr (Table 2, results obtained at 0 hr).

No significant change in the extent of parasite interaction with untreated MPM was seen when the trypomastigotes were pretreated with up to 100 µg LF/ml for 2 hr (data not shown). This negative result led us to test whether or not the parasite would bind LF. The results of indirect immunofluorescence tests failed to produce any evidence of such binding when the flagellates were incubated with up to 100 µg LF/ml.

Effects of LF on trypomastigote killing by MPM or HBM. The results of experiments in which the MPM were pretreated with 10 µg LF/ml for 2 hr, washed, and then exposed to *T. cruzi* for 2 hr in the absence of LF, showed that the number of parasites per 100 MPM was significantly smaller than that of mock treated MPM when measured 70 hr later, but not 22 or

TABLE 1 - MINIMAL PRETREATMENT TIME OF MPM WITH LF REQUIRED FOR PRODUCTION OF ENHANCED UPTAKE AND KILLING OF BLOOD FORMS OF *T. cruzi*

LF (10µg/ml)	Time* (h)	0 h* % MPM with parasites (%C)†	0 h* No. parasites per 100 MPM (%C)	10 h* % MPM with parasites (%R)§	10 h* No. parasites per 100 MPM (%R)	22 h* % MPM with parasites (%R)	22 h* No. parasites per 100 MPM (%R)
Absent		24.1 ± 1.4	30.5 ± 1.3	21.6 ± 2.0	29.0 ± 4.3	20.0 ± 1.9	26.5 ± 5.6
Present	6	19.0 ± 0.9	23.0 ± 2.5	24.8 ± 1.0	28.3 ± 0.7	20.1 ± 3.6	26.1 ± 4.3
Absent		23.3 ± 4.2	28.8 ± 3.4	21.3 ± 1.4	27.5 ± 2.2	20.1 ± 2.2	24.0 ± 2.1
Present	12	24.6 ± 0.3	35.5 ± 1.8 (23)	16.8 ± 2.0 (32)	18.6 ± 2.7 (47)	12.8 ± 1.5 (48)	15.3 ± 2.3 (57)
Absent		21.1 ± 2.0	26.3 ± 3.7	21.6 ± 1.5	27.1 ± 0.6	18.7 ± 1.2	23.2 ± 0.7
Present	24	42.1 ± 4.6(100)	54.6 ± 6.8 (108)	16.0 ± 2.8 (62)	19.1 ± 3.2 (55)	13.5 ± 2.5 (68)	15.8 ± 2.7 (71)

For footnotes please see next page

Footnotes to table 1

* MPM were incubated with LF for 6, 12 or 24 h, washed and cultured with the parasites for 2 h. After removing the non-bound organisms, one third of the MPM cultures were fixed whereas the remaining cultures received fresh medium and were incubated for an additional 10 or 22 h.

† (%C), percent increase in parasite uptake by LF-treated MPM (calculated with respect to the value obtained in the absence of LF). A %C value is shown where it was statistically significant ($P \leq 0.05$).

§ {%R}, percent reduction in parasite load calculated with respect to the corresponding value obtained immediately after removing the non-bound organisms (0-h values); a %R value is shown where it was statistically significant ($P \leq 0.05$).

TABLE 2 - EFFECTS OF PRETREATMENT OF HBM WITH LF ON THEIR CAPACITY TO TAKE UP AND KILL BLOOD FORMS OF *T. cruzi*

	0 h		10 h	
Treatment of HBM*	% HBM with parasites (%C)†	No. parasites per 100 HBM (%C)	% HBM with parasites (%R)§	No. parasites per 100 HBM (%R)
DMEM+FBS	18.2 ± 3.2	19.4 ± 2.8	15.0 ± 0.7	19.5 ± 2.1
LF, 10 µg/ml	26.0 ± 2.1 (43)	30.5 ± 2.8 (57)	10.5 ± 0.7 (60)	13.7 ± 5.3 (55)
LF, 100 µg/ml	40.2 ± 3.9 (121)	53.0 ± 6.4 (173)	6.0 ± 1.4 (85)	7.5 ± 3.5 (87)

* HBM were incubated with LF for the 1 h, washed and cultured with the parasites for 2 h. After removing the non-bound organisms, half of the HBM cultures were fixed immediately (0 h) whereas the other half received fresh medium and were incubated for an additional 10 h before termination.

†,§ See footnotes under Table 1.

46 hr later (Table 3).However, the percentages of infected MPM showed insignificant variations during the 70-hr period. Similar results were obtained when 10 µg LF/ml was present in the MPM cultures during the 2-hr period that these cells were exposed to the trypomastigotes (Fig. 1). Because in our experimental system free parasites usually appear in the culture medium approximately 4 days after MPM infection (Lima and Kierszenbaum, unpublished results), and this would have complicated the interpretation of the results, measurements were not made after 70 hr.

In addition to enhancing parasite uptake by MPM or HBM, pretreatment of MPM with 10 µg LF/ml for 24 hr and of HBM with 10 or 100 µg LF/ml for 2 hr increased their cytotoxic capacities (Table 1, results obtained after 10 or 22 hr and Table 2, results obtained after 10 hr). As seen in Table 1, the reductions in the parasite contents of LF-treated MPM over the 10- and 22-hr incubation periods after the removal of free flagellates were significantly greater than those effected by MPM which had been mock treated with medium alone. Similar results were obtained when HBM were used (Table 2). Two alternative explanations were compatible with these observations: LF-treated cells could destroy more parasites simply because they had initially taken up larger numbers of organisms or because they had been activated. To test these possibilities, we measured the effect of LF on the cytotoxic capacity of MPM after parasite internalization. The results revealed that substantial parasite growth had occurred in the mock-treated MPM over a 72-hr period whereas the LF-treated MPM were able not only to contain growth but also to destroy many organisms (Table 4).

Mechanism of parasite killing. Killing of the intracellular, amastigote

TABLE 3 - VARIATIONS IN THE PARASITE CONTENTS OF MPM AFTER LF PRETREATMENT FOR 2 h

LF pretreatment[a]	Time* (h)	% MPM with parasites	No. parasites per 100 MPM {%R}†
No	0	33.1 ± 2.7	36.0 ± 1.8
Yes	0	30.2 ± 2.5	33.8 ± 1.2
No	22	25.3 ± 1.2	29.0 ± 1.3
Yes	22	25.0 ± 1.5	27.1 ± 2.0
No	46	25.2 ± 2.2	51.3 ± 3.5 {-43}
Yes	46	24.6 ± 4.0	49.0 ± 0.5 {-45}
No	70	24.2 ± 2.7	105.5 ± 5.6 {-193}
Yes	70	23.1 ± 1.8	45.1 ± 3.8 { -33}

For footnotes please see next page

Footnotes to Table 3

* The MPM cultures were incubated with 10 µg LF/ml for 2 h, washed and then incubated with trypomastigotes for 2 h. After removing the free organisms, some cultures were fixed (0 h) and the rest received fresh DMEM+FBS and were further incubated for the indicated periods of time.

† See footnote § under Table 1.

FIGURE LEGEND

FIG. 1. Internalization of *T. cruzi* trypomastigotes by MPM in the presence (■) or absence (●) of 10 µg LF/ml. LF was present during the 2-h parasite-MPM co-culture, then removed by washing, and the MPM were further incubated in fresh medium for the indicated periods of time. Points represent the mean of triplicate determinations and bars the standard deviation. Only the difference shown on the right panel for 70 h was statistically significant ($P \leq 0.05$).

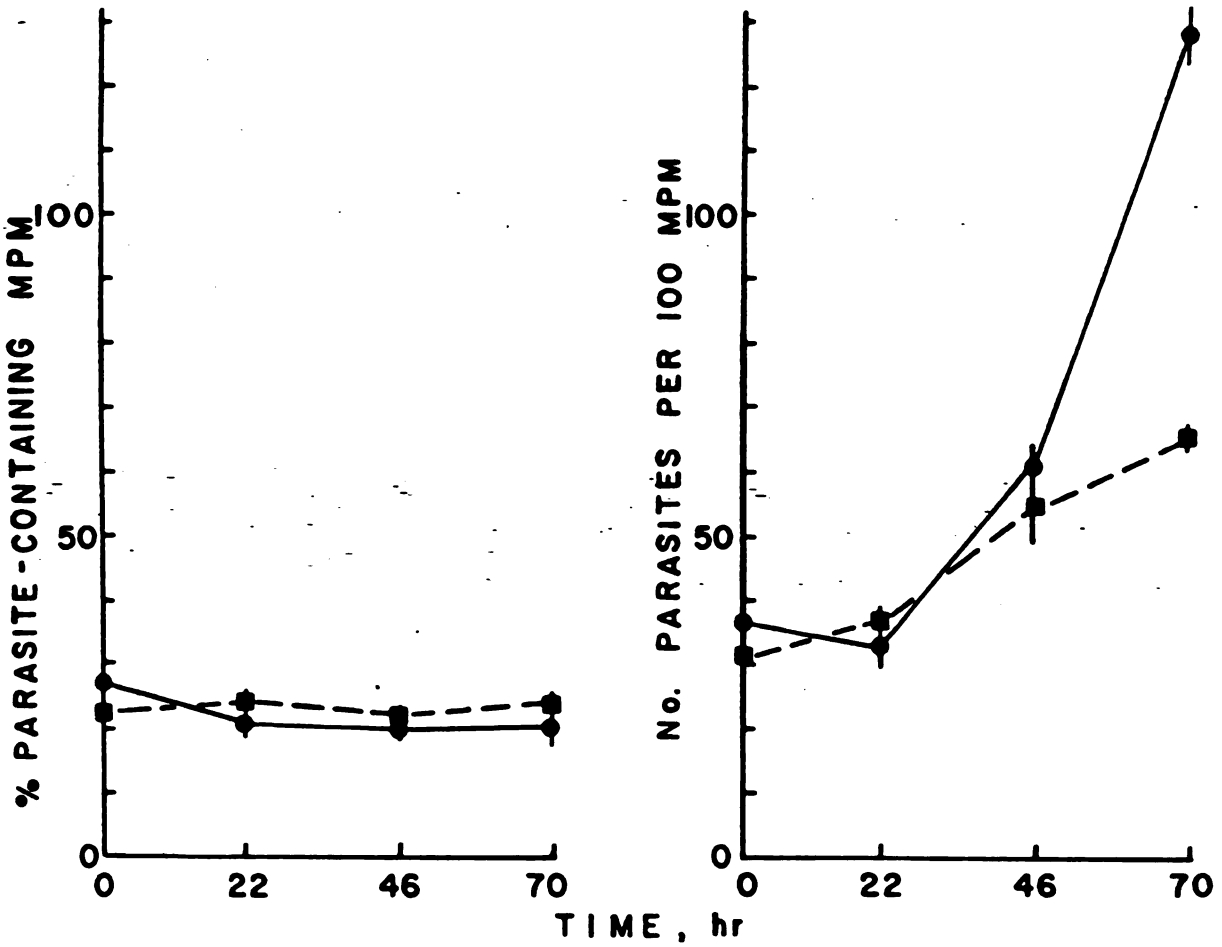
% PARASITE-CONTAINING MPM
100
50
0
0
22
46
70
No. PARASITES PER 100 MPM
100
50
0
0
22
46
70
TIME, hr

Figure 1

TABLE 4 - EFFECTS OF TREATMENT OF MPM WITH LF AFTER INFECTION WITH *T. cruzi*

Treatment*	Length of treatment (h)	% MPM with parasites (%R)†	No. parasites per 100 MPM (%R)
None		33.5 ± 1.8	47.3 ± 6.4
DMEM+FBS	24	26.0 ± 2.2	42.8 ± 7.3
LF	24	12.6 ± 1.8 (65)	15.3 ± 1.9 (68)
DMEM+FBS	48	29.6 ± 1.1	76.1 ± 8.2 (-61)§
LF	48	13.0 ± 2.6 (63)	21.8 ± 3.5 (54)
DMEM+FBS	72	27.2 ± 1.7	127.5 ±11.3 (-170)§
LF	72	9.8 ± 2.0 (72)	20.6 ± 7.5 (56)

For footnotes please see next page

Footnotes to Table 4

* MPM monolayers were incubated with the parasites for 2 h and washed to remove the non-bound organisms. Whereas some cultures were immediately terminated by fixation (top row of values), the remainder received either DMEM+FBS or the same medium containing 10 µg LF/ml, and were further incubated for the indicated periods of time before termination.

† See footnote § under Table 1.

§ A negative %R represents an increase in the parasite load of MPM.

form of T. cruzi by unstimulated MPM was previously found to be mediated by H_2O_2 (Villalta & Kierszenbaum, 1984a). To find out if LF-treated MPM killed the blood forms of this parasite by a similar mechanism, MPM were first pretreated with 10 µg LF/ml for 24 hr, then incubated with the flagellates for 2 hr, and later on washed with and incubated in fresh medium for an additional 24 hr, having catalase -a scavenger of H_2O_2- present or absent during these steps. As can be seen in Table 5, catalase, but not heat-inactivated catalase, markedly inhibited parasite killing by LF-stimulated MPM. Catalase and heated catalase had no effect of their own on parasite internalization by MPM whether or not these cells had been treated with LF.

TABLE 5 - EFFECTS OF THE PRESENCE OF CATALASE ON THE CAPACITY OF LF-TREATED MPM TO KILL BLOOD FORMS OF *T. cruzi*

Treatment of MPM*	0 h*		22 h*	
	% MPM with parasites (%C)†	No. parasites per 100 MPM (%C)	% MPM with parasites (%R)§	No. parasites per 100 MPM (%R)
DMEM+FBS	12.5 ± 2.8	13.2 ± 3.8	10.5 ± 2.0	10.8 ± 2.4
LF, 10 µg/ml	22.2 ± 2.5 (78)	23.7 ± 3.0 (80)	4.2 ± 0.7 (81)	4.7 ± 2.5 (80)
LF + catalase	22.5 ± 2.1 (80)	24.0 ± 2.1 (82)	21.7 ± 0.5	27.2 ± 2.2
Catalase	13.0 ± 0.7	14.7 ± 0.3	14.1 ± 1.0	15.2 ± 0.7
HI-catalase	13.7 ± 1.7	14.5 ± 2.1	14.0 ± 1.0	15.2 ± 0.7
LF+HI-catalase	22.5 ± 3.5 (80)	23.7 ± 3.1 (80)	4.2 ± 0.2 (81)	4.2 ± 0.2 (82)

For footnotes please see next page

Footnotes to Table 5

* MPM monolayers were treated with LF in the presence or absence of catalase or heat-inactivated catalase (HI-catalase) for 24 h, washed and incubated further with T. cruzi for 2 h in the presence or absence of catalase or HI-catalase. After removing the non-bound organisms, the cultures were either fixed immediately (0 h) or incubated for an additional 22 h with fresh medium containing catalase or HI-catalase before termination.

†,§ See footnotes under Table 1.

DISCUSSION

The conditions inducing increased trypomastigote uptake varied depending on whether MPM or HBM were used. MPM had to be pretreated with LF for at least 24 hr for the phenomenon to be seen (Table 1) whereas HBM required only a 2-hr pretreatmnet (Table 2). Van Snick and Masson (1976) determined the average number of LF receptors per MPM to be 2×10^7 per cell and Bennett and Davis (1981) found the corresponding value for HBM to be 2×10^8; the presence of a greater number of LF receptors on the HBM could explain why these cells responded faster than MPM to LF in terms of increased parasite uptake.

The number of receptors for LF on the trypomastigotes, must be relatively small, if any, because indirect immunofluorescence tests to detect bound LF on these organisms were systematically negative. The lack of receptors for LF on trypomastigotes and the fact that pretreatment of this parasite form did not alter its uptake by untreated MPM rendered unlikely the possibility that LF could have increased parasite uptake by establishing a molecular bridge between the two cells.

Whether LF was present during the 2-hr period of MPM-*T. cruzi* interaction or the MPM were pretreated with LF for 2 hr, the percentages of MPM with parasites did not vary significantly during the 70-hr observation period (Fig. 1 and Table 3). Instead, the number of parasites per 100 LF-treated MPM was significantly reduced during the same period of time. These findings, viewed in the light of the concomitant increase in the parasite load of mock-treated MPM, indicated that LF-treated MPM had an increased capacity to kill *T. cruzi* trypomastigotes. Indicating that

the reduction in parasite load fostered by LF involved parasite killing was the ability of catalase to inhibit this effect (Table 5). This enzyme has been shown to inhibit trypomastigote destruction by MPM stimulated by interferon gamma (Wirth, Kierszenbaum, Sonnenfeld & Zlotnik, 1985). The development of enhanced cytotoxicity by LF-treated MPM was time-dependent, requiring preincubation with 10 µg LF/ml for a period of 12 to 24 hr before being detectable (Table 1). However, HBM developed a similar capacity after only 2-hr of pretreatment with the same concentration of LF (Table 2). This kinetic difference might also be a function of the density of LF receptors on the surface of phagocytic cells alluded to above.

When the MPM were pretreated with LF for 2 hr, a reduction in their parasite load was seen 70 hr after removal of the free parasites (Table 3). However, this effect occurred much earlier (i.e., after 10 hr) when the MPM were pretreated with LF for 24 hr (Table 1). While these results stress the stimulatory effect of LF on MPM cytotoxicity, they do not provide a definitive explanation for the noted kinetic difference.

Trypomastigotes were also destroyed at faster rates when MPM which had already ingested the flagellates were treated with LF (Table 4). This finding reinforces that LF increases the cytotoxic capacity of MPM and indicates that enhanced killing was not necessarily dependent on the initial uptake of a larger number of organisms. The involvement of H_2O_2 in parasite killing by LF-treated MPM was denoted by the inhibitory effect of catalase (Table 5). The results, however, do not clarify whether H_2O_2 was directly toxic for the parasite or acted indirectly, via production of other toxic O_2 reduction intermediates.

Of interest in this context is that H_2O_2 is also involved in trypomastigote killing by MPM when these cells are stimulated with interferon gamma (Wirth et al., 1985). Thus, H_2O_2 would play a role in T. cruzi destruction within MPM following activation by different stimuli.

Unlike amastigotes (Lima & Kierszenbaum, 1985), trypomastigotes did not bind LF. Furthermore, the macrophages required a longer (24 hr) pretreatment with LF to display enhanced uptake and killing of trypomastigotes than to achieve similar effects with amastigotes (1 hr). These differences, for which we can not provide an explanation at the present time, are possibly related to the distinct biological features of these two life cycle stages of T. cruzi.

The noted activities of LF might be related to the level of iron saturation, its cationic property, or to both. Finally, it is noteworthy that the concentrations of LF found to enhance phagocyte interaction with T. cruzi are within the range found in the plasma of patients with inflammatory conditions (Hansen, Karle, Andersen, Malmquist & Hoff, 1976; Zenian & Kierszenbaum, 1982; Lima & Kierszenbaum, 1985) similar to those occurring in T. cruzi infection and might be involved in host defense. These possibilities deserve further study.

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REFERENCES

ALCANTARA, A. & BRENER, Z. 1980. Trypanosoma cruzi: role of macrophage membrane components in the phagocytosis of bloodstream forms. Experimental Parasitology 50:1-6.

ANDRADE, Z. A. & ANDRADE, S. G. 1979. Patologia. In: Trypanosoma cruzi e Doenca de Chagas, pp. 199-248 (Edited by Brener, Z. & Andrade, Z. A.) Guanabara Koogan, Rio de Janeiro.

BENNETT, R. M. & DAVIS, J. 1981. Lactoferrin binding to human peripheral blood cells: an interaction with a β- enriched population of lymphocytes and a subpopulation of adherent mononuclear cells. Journal of Immunology 127:1211-1216.

BUDZKO, D. B. & KIERSZENBAUM, F. 1974. Isolation of Trypanosoma cruzi from blood. Journal of Parasitology 60:1037-1038.

CARVALHO, R. M., MEIRELLES, M. N. L., DE SOUZA, W. & LEON, W. 1981. Isolation of the intracellular stage of Trypanosoma cruzi and its interaction with mouse macrophages in vitro. Infection and Immunity 33:546-554.

CONNELLY, M. C. & KIERSZENBAUM, F. 1984. Modulation of macrophage interaction with Trypanosoma cruzi by Phospholipase A_2-sensitive components of the parasite membrane. Biochemical and Biophysical Research Communications 121:931-939.

CONNELLY, M. C. & KIERSZENBAUM, F. 1985. Increased host cell-Trypanosoma cruzi interaction following Phospholipase D treatment of the parasite surface. Molecular and Biochemical Parasitology 17:191-202.

DE ALMEIDA MARIA, T., ALCANTARA, A. & BRENER, Z. 1982. Ultrastructural studies on the in vitro interaction of Trypanosoma cruzi bloodstream forms and mouse peritoneal macrophages. Acta Tropica 39:99-109.

HANSEN, N. E., KARLE, H., ANDERSEN, V., MALQUIST, J. & HOFF, G. E. 1976. Neutrophilic granulocytes in acute bacterial infection. Sequential studies on lysozyme, myeloperoxidase and lactoferrin. Clinical and Experimmental Immunology 26:463-468.

KIERSZENBAUM, F. 1979. Antibody-dependent killing of bloodstream forms of Trypanosoma cruzi by human peripheral blood leukocytes. American Journal of Tropical Medicine and Hygiene 28:965-968.

KIERSZENBAUM, F. & HAYES, M. M. 1980. Mechanisms of resistance against experimental Trypanosoma cruzi infection. Requirements for cellular destruction of circulating forms of T. cruzi in human and murine in vitro systems. Immunology 40:61-66.

KIERSZENBAUM, F., KNECHT, E., BUDZKO, D. B. & PIZZIMENTI, M. C. 1974. Phagocytosis: a defense mechanism against infection with Trypanosoma cruzi. Journal of Immunology 112:1839-1844.

KIERSZENBAUM, F., VILLALTA, F. & TAI, P.-C. 1986. Role of inflammatory cells in Chagas' disease. III. Kinetics of human eosinophil activation upon interaction with parasites (Trypanosoma cruzi). Journal of Immunology 136:662-666.

LIMA, M. F. & KIERSZENBAUM, F. 1985. Lactoferrin effects on phagocytic cell function. I. Increased uptake and killing of an intracellular parasite by murine macrophages and human monocytes. Journal of Immunology 134:4176-4183.

NATHAN, C., NOGUEIRA, N., JUNAGBHANICH, C., ELLIS, J. & COHN, Z. 1979. Activation of macrophages in vivo and in vitro. Correlation between hydrogen peroxide release and killing of Trypanosoma cruzi. Journal of Experimental Medicine 149:1056-1068.

OKABE, K., KIPNIS, T. L., CALICH, V. L. G. & DIAS DA SILVA, W. 1980. Cell-mediated cytotoxicity to Trypanosoma cruzi. I. Antibody-dependent cell-mediated cytotoxicity to trypomastigote bloodstream forms.Clinical Immunology and Immunopathology 16:344-353.

PRYSWANSKY, K. B., MACRAE, E. K., SPITZNAGEL, J. K. & COONEY, M. H. 1979. Early degranulation of human neutrophils: immunocytochemical studies of surface and intracellular phagocytic events. Cell 18:1025-1033.

ROMAÑA, C. 1963. In: Enfermedad de Chagas, pp. 97-146 (Edited by Romaña,C.) Lopez Libreros Editores, Buenos Aires.

SCOTT, M. T. & SNARY, D. 1982. American trypanosomiasis (Chagas'disease).In: Immunology of Parasitic Infections, pp. 261-298 (edited by Cohen, S. & Warren, K. S.) Blackwell Scientific Publications, Oxford.

SPITZNAGEL, J. K. 1984. Nonoxidative antimicrobial reactions of leukocytes.In: Regulation of Leukocyte Function, pp. 283-343 (Edited by Snyderman,R.) Plenum Press, New York.

VAN SNICK, J. R. & MASSON, P. L. 1976. The binding of human lactoferrin to mouse peritoneal cells. Journal of Experimental Medicine 144:1568-1580.

VILLALTA, F. & KIERSZENBAUM, F. 1983. Role of polymorphonuclear cells in Chagas' disease. I. Uptake and mechanisms of destruction of intracellular (amastigote) forms of Trypanosoma cruzi by human neutrophils. Journal of Immunology 131:1504-1510.

VILLALTA, F. & KIERSZENBAUM, F. 1983a. Role of cell surface mannoseresidues in host cell invasion by Trypanosoma cruzi. Biochimica et Biophysica Acta 736:39-44.

VILLALTA, F. & KIERSZENBAUM, F. 1984. Role of inflammatory cells in Chagas'disease. I. Uptake and mechanisms of destruction of intracellular(amastigote) forms of Trypanosoma cruzi by human eosinophils. Journal of Immunology 132:2053-2058.

VILLALTA, F. & KIERSZENBAUM, F. 1984a. Role of inflammatory cells in Chagas' disease. II. Interactions of mouse macrophages and human monocytes with intracellular forms of Trypanosoma cruzi: uptake and mechanism of destruction. Journal of Immunology 133:3338-3343.

VILLALTA, F. & KIERSZENBAUM, F. 1986. Effects of human colony-stimulating factor on the uptake and destruction of a pathogenic parasite(Trypanosoma cruzi) by human neutrophils. Journal of Immunology 137:1703-1707.

VILLALTA, F. & LEON, W. 1979. Effect of purification by DEAE-cellulose on infectivity of Trypanosoma cruzi blood forms. Journal of Parasitology 65:188-189.

WIRTH, J. J. & KIERSZENBAUM, F. 1982. Inhibitory action of elevated levels of adenosine-3':5' cyclic monophosphate on phagocytosis: Effects on macrophage-Trypanosoma cruzi interaction. Journal of Immunology 129:2759-2762.

WIRTH, J. J. & KIERSZENBAUM, F. 1983. Modulatory effect of guanosine-3':5'cyclic monophosphate on macrophage susceptibility to Trypanosoma cruzi infection. Journal of Immunology 121:3028-3031.

WIRTH, J. J. & KIERSZENBAUM, F. 1984. Fibronectin enhances macrophage association with invasive forms of Trypanosoma cruzi. Journal ofImmunology 133:460-464.

WIRTH, J. J., KIERSZENBAUM, F., SONNENFELD, G & ZLOTNIK, A. 1985. Enhancing effects of gamma interferon on phagocytic cell association with and killing of Trypanosoma cruzi. Infection and Immunity 49:61-66.

WOLACH, B., COATES, T. D., HUGLI, T. E., BAEHNER, R. L. & BOXER, L. A. 1984. Plasma lactoferrin reflects granulocyte activation in burn patients. Journal of Clinical and Laboratory Medicine 103:284-293.

ZENIAN, A. & KIERSZENBAUM, F. 1982. Inhibition of macrophage-Trypanosoma cruzi interaction by concanavalin A and differential binding of bloodstream and culture forms to the macrophage surface. Journal of Parasitology 68:408-415.

CHAPTER III

LACTOFERRIN EFFECTS ON PHAGOCYTIC CELL FUNCTION. DISTINCT ROLES IN MODULATION OF MACROPHAGE INTERACTION WITH A PARASITE (*Trypanosoma cruzi*)

ABSTRACT

The present studies on the role of iron in the ability of lactoferrin (LF) -a neutrophil glycoprotein whose levels in body fluids increase in inflammatory conditions- to stimulate macrophage functions unveiled two distinct modes of action involved in modifying uptake and killing. The presence of iron was not a requirement for LF to enhance the capacity of mouse peritoneal macrophages (MPM) to take up Trypanosoma cruzi amastigotes (AMA) or latex particles. In contrast, iron was required to be in the molecule in order to stimulate AMA killing by the MPM. Thus, ApoLF was incapable and LF lost its ability to enhance AMA killing in the presence of the iron chelator deferoxamine. Similar results were obtained by using diethylenetriaminepentaacetic acid, an iron chelator which is not incorporated into cells, suggesting that iron had to be a part of the LF molecule while LF was on the MPM membrane. Moreover, ApoLF did increase MPM killing of AMA when ferric ions were restored to the molecule. Such restoration was demonstrable also when iron was added to the culture medium after MPM treatment with, and removal of the non-bound, ApoLF. Interestingly, ferrous and ferric ions were equally effective in restoring activity to ApoLF and, though to a lesser extent, also zinc. Transferrin did not alter the capacity of MPM to either take up or kill the AMA, suggesting that the noted LF effects were probably specific for the latter protein. Immunofluorescence studies revealed that ApoLF and LF at either 20 or 100% iron saturation bound to approximately the same proportion of MPM, and that the fluorescence intensity of positive cells was comparable in all three cases. Thus, the lack of

effect of ApoLF on MPM killing is unlikely to be due to ApoLF not binding to MPM. Killing of internalized AMA by LF-treated MPM, known to be mediated at least in part by H_2O_2, $O_2^{\cdot-}$ and $^1O_2$, was found in this work to involve also OH$^{\cdot}$ While providing information concerning the requirements for LF to enhance macrophage killing of T. cruzi, these results also evidence a clear separation of the uptake and killing functions of macrophages which depends on the activating stimulus.

INTRODUCTION

Lactoferrin (LF), is a typical example of a secretion product of an inflammatory-type cell, the neutrophil (1; reviewed in reference 2), which can modulate the functions of another inflammatory cell. For example, LF has been reported to inhibit the production of colony-stimulating factor by human monocyte-derived (3) and mouse (4, 5) macrophages, and to increase monocyte tumoricidal activity (6). We previously reported that treatment with lactoferrin (LF) increases the capacity of mouse resident peritoneal macrophages (MPM) to interact with amastigote forms of Trypanosoma cruzi (AMA), the causative agent of Chagas' disease (7). In this case, the LF effects translated into greater uptake of this parasite, followed by killing at a significantly faster than normal rate. This model system of macrophage interaction with a microorganism was used in the present work to explore the molecular requirements for LF to evoke the above-mentioned effects. LF is an iron-binding protein (reviewed in references 8 and 9) and the presence or absence of iron ions in the molecule has been shown to be an important factor in determining its ability to inhibit colony-stimulating factor production by human macrophages (3, 5). For these reasons, we examined whether iron was a requirement for LF to enhance the uptake and killing of T. cruzi by MPM. The results presented in this paper established that the presence or absence of iron in the LF molecule affords a clearcut distinction between these two macrophage functions that is emphasized by different molecular requirements.

MATERIALS AND METHODS

Parasites. Tulahuén strain T. cruzi amastigotes were prepared as described in detail previously (10). Parasite suspensions at 2.5 X 10^7 organisms/ml were prepared in minimal essential medium with Hank's salts containing 100 IU penicillin and 100 µg streptomycin per ml (MEM) plus 1% BSA (MEM+BSA). All of the suspensions used in this work consisted exclusively of amastigotes (>99% viable).

Reagents. Human LF was purchased from Sigma Chemical Co. (St. Louis, MO) and was verified to be 20% iron saturated as described by Ambruso and Johnston (11). Iron-free LF (ApoLF) was prepared by first incubating a mixture containing 500 µg/ml LF and 0.25M deferoxamine (Ciba-Geigy, Summit, NJ) in phosphate-buffered saline pH 7.2 (PBS) at 37°C for 1 hr; deferoxamine was removed by dialysis at 4°C vs. PBS. This material was determined to be iron-free. LF was saturated with ferric ions by incubating a mixture containing 1 mg/ml LF in PBS with 1mM ferric citrate at room temperature for 24 hr. Excess iron ions were removed by dialysis vs. PBS (4°C, 24 hr, three changes of PBS). The solutions of these proteins were aliquoted and stored at -20°C until used. Human Transferrin at 0% and 100% iron saturation were purchased from Calbiochem (La Jolla, CA). All necessary dilutions of these reagents were made in MEM+BSA.

Mouse peritoneal macrophages (MPM). The methods to obtain MPM from Crl:CD-1(ICR)BR Swiss mice (Charles River Laboratory, Portage, MI) and to prepare monolayers of these cells have been described elsewhere(7).

The cultures routinely consisted of >98% nonspecific-esterase positive cells with typical macrophage morphology.

Pretreatment of MPM with LF or ApoLF. Monolayers of MPM were incubated at 37°C for 1 hr with 0.2 ml of MEM+BSA alone or containing 10 µg/ml of Apolactoferrin (ApoLF) or LF at 20% or 100% iron saturation. The monolayers were then washed three times with MEM and used in the assays to determine macrophage-parasite interactions (see below).

Determination of MPM association with AMA. All experimental and control conditions were tested in triplicate. MPM cultures treated with either LF or MEM+BSA received 0.1 ml of AMA suspension plus 0.2 ml of MEM+BSA and were incubated at 37°C in a 5% CO_2-in-air atmosphere for 2 hr. After removing the free organisms by five washings with MEM, the cultures were fixed with absolute methanol and stained with Giemsa. At least 200 cells were examined microscopically (X1000) on each culture. The number of parasites associated with these cells and the number of MPM with and and without parasites were recorded and used to calculate the mean percentage of MPM with parasites and the average number of organisms per 100 MPM.

Determination of MPM killing of AMA. A replicate set of cultures initiated as described above was further incubated -after removing the non-bound AMA- with 0.3 ml of MEM supplemented with 10% heat-inactivated (56°C, 1 hr) FBS dialyzed vs. PBS (4°C, 24 hr) (MEM+dFBS) at 37°C (5% CO_2) for an additional 6 hr. Termination and processing of these cultures were as described above.

In experiments designed to test the iron dependency of the observed LF effects on MPM function, MPM were simultaneously incubated

with LF and various concentrations of deferoxamine (see Results) at 37°C for 1 hr. These reagents were washed off with MEM prior to exposing the MPM to AMA.

To investigate the mechanism(s) underlying our observations, a protocol was used in which deferoxamine, diethylenetriaminepentaacetic acid (DTPA), urea, thiourea or α-ketobutyric acid was present in the medium from 2 hr after placing the MPM into the culture chambers until termination of the experiment.

In experiments designed to test the effect of cation restoration of the LF effects on MPM association with and killing of AMA, the relevant cation (see Results) was added to the culture medium immediately after MPM pretreatment with ApoLF and the cultures were further incubated for 1 hr. After washing three times with MEM, the parasites were added. The rest of the experiment proceeded as described above for determination of both MPM association with and killing of AMA.

Indirect immunofluorescence assay for LF. The method to establish LF binding to both MPM and AMA has been described (7).

Latex bead uptake by ApoLF- or LF-treated and mock-treated MPM. Cultures of MPM were incubated at 37°C (5% CO_2) for 1 hr with ApoLF or with LF at 20% or 100% iron saturation and washed three times with MEM before receiving 0.3 ml of latex bead suspension (8.3×10^6 beads/ml MEM+BSA; average bead diameter 1 μm, Sigma). After further incubation for 2 hr and removal of the free beads, the cultures were fixed with methanol, stained with Giemsa and examined microscopically (X1000) to establish the percentage of MPM with latex beads and the number of beads per 100 MPM.

Presentation of results and statistics. All results are expressed as the mean $\pm$ 1 S.D. Differences were considered to be significant if $P \leq 0.05$ as determined by the Mann-Whitney "U" test. The sets of results presented in the tables are typically representative of two to three separate repeat experiments.

RESULTS

Effects of iron content on the capacity of LF to enhance MPM association with or killing of AMA. In previous work, we showed that LF at 20% iron saturation enhanced the capacity of MPM to associate with and kill T. cruzi AMA (7). In the present work, this effect was reproduced and, furthermore, found to be also induced by LF at 100% iron saturation (Table I). However, iron removal from the molecule prior to its use in the treatment of MPM virtually abrogated the enhanced killing effect but failed to alter the capacity of these cells to associate with AMA. ApoLF recovered its capacity to enhance parasite killing after iron replacement, achieved by mixing ApoLF with ferric citrate (excess iron ions were removed by dialysis). Ferric ions alone, in the absence of ApoLF, had no detectable consequence on the extent of either MPM-parasite association or killing. To confirm the iron requirement for LF to enhance AMA killing, we used an alternative approach in which the MPM were incubated with LF in the presence of the iron chelator deferoxamine and then exposed to the parasites. A representative set of results is presented in Table II, showing that the extent of parasite killing by LF-treated MPM was reduced from 73% to 44% and 10% in the presence of 0.5mM and 5mM deferoxamine, respectively. The lowest concentration of deferoxamine tested, 0.05mM, was ineffective in curtailing the stimulatory effect of LF on parasite destruction. Deferoxamine alone had no detectable effect on either MPM association with and killing of AMA at any of the tested concentrations. In some experiments, we used DTPA, an iron-chelating agent which, unlike deferoxamine, can not enter cells (12). As shown in Table III, DTPA was as effective in inhibiting the LF-induced enhancement of AMA killing as

Table I

Effects of iron content on the ability of LF to enhance MPM association with and killing of T. cruzi[a]

Macrophage pretreatment	No. of AMA per 100 MPM after 2 hr	8 hr
MEM+BSA	28.8 ± 2.8	27.1 ± 4.1
LF, 20% iron saturation	56.5 ± 7.5[b]	27.8 ± 1.6 [28.7] (51)
LF, 100% iron saturation	67.6 ± 9.9[b]	20.0 ± 4.5 [47.6] (70)
ApoLF	57.0 ± 3.6[b]	53.3 ± 3.2
Ferric citrate-treated ApoLF	58.3 ± 4.7[b]	20.5 ± 1.5 [37.8] (65)
Ferric citrate, 2.6 μM	28.8 ± 4.9	24.0 ± 2.3

For footnotes please see next page

Footnotes to Table I

[a]Cultures of MPM were incubated with the solution of the indicated material(s) at 37°C for 1 hr, washed with MEM and then incubated with AMA for 2 hr. Half of the cultures were then terminated by fixation whereas the other half received MEM+dFBS and was incubated for an additional 6 hr. ApoLF or LF were used at 10 µg/ml.

[b]The difference between this value and the corresponding control was statistically significant ($P \leq 0.05$). Values in brackets represent the actual decrease in the average number of parasites per 100 MPM. Values in parentheses represent the percentage of reduction in parasite contents occurring over the 6-hr time interval. Values in brackets and parentheses are shown only where the difference was statistically significant with respect to the corresponding 2-hr value.

Table II

Effects of iron chelation on the ability of LF to enhance MPM killing of T. cruzi[a]

Macrophage pretreatment	No. of AMA per 100 MPM after 2 hr	8 hr
MEM+BSA	31.1 ± 4.0	27.8 ± 1.2
LF	57.6 ± 0.7[b]	15.8 ± 2.5 [41.8] (73)
LF + 0.05mM deferoxamine	56.0 ± 7.7[b]	17.5 ± 0.5 [38.5] (69)
LF + 0.5mM deferoxamine	55.6 ± 7.5[b]	31.1 ± 2.3 [24.5] (44)
LF + 5mM deferoxamine	55.1 ± 2.2[b]	49.8 ± 3.0
Deferoxamine 0.05mM	28.6 ± 3.2	28.5 ± 2.5
Deferoxamine 0.5mM	27.1 ± 3.7	24.6 ± 3.6
Deferoxamine 5mM	29.6 ± 5.0	27.5 ± 2.6

[a,b] See footnotes to Table I.

Table III

Effects of DTPA or deferoxamine on the ability of LF to enhance MPM killing of T. cruzi[a]

Macrophage pretreatment	No. of AMA per 100 MPM after 2 hr	8 hr
MEM+BSA	19.0 ± 1.5	21.1 ± 3.4
LF	36.3 ± 1.0[b]	8.6 ± 0.6 [27.7] (76)
LF + 0.15mM DTPA	37.3 ± 3.7[b]	37.8 ± 2.5
DTPA	17.6 ± 0.3	22.8 ± 1.6
LF + 5mM deferoxamine	36.8 ± 2.7[b]	39.0 ± 0.8
Deferoxamine	17.0 ± 2.1	18.5 ± 3.1

For footnotes please next page

Footnotes to table III

[a]When designed to be present, DTPA or deferoxamine was added to the culture medium 2 hr after placing the MPM in the culture chambers and remained present until the end of the experiment, including washings. Otherwise, the protocol was as described in the footnote to Table I.

[b]See footnote to Table I.

deferoxamine. It is noteworthy that, under the tested conditions, neither deferoxamine nor DTPA had any detectable effect on MPM or AMA viability as determined by trypan-blue exclusion and vibratile *in situ* motion, respectively. When results obtained in repeat experiments were compared, the extents of the noted effects sometimes varied. However, the effects themselves were always readily reproducible and statistically significant.

We considered the possibility that the absence of iron in the LF molecule might have affected its ability to be bound by MPM and conducted several types of experiments to test it. When MPM were incubated with solutions containing the same concentrations of ApoLF, LF at 20% iron saturation and LF at 100% iron saturation, the percentages of cells binding these proteins, determined by immunofluorescence, were 76 $\pm$ 4, 84 $\pm$ 6 and 86 $\pm$ 8 percent, respectively; the control value obtained with MPM incubated with MEM+BSA alone was 0 $\pm$ 0 percent. The intensity of the fluorescence of the positive MPM was comparable in all three cases. Moreover, MPM pretreated with ApoLF, LF at 20% iron saturation and LF at 100% iron saturation increased the capacity of MPM to ingest latex beads similarly (from a control level of 57 $\pm$ 3 percent to 125 $\pm$ 11, 107 $\pm$ 11 and 135 $\pm$ 2 percent, respectively). This had also been the case with AMA uptake (Table I). In an alternative approach, we incubated the MPM first with ApoLF and, after washing the cells exhaustively, incorporated ferric ions into the system in an amount sufficient to have saturated the initial amount of LF. The result was enhanced AMA killing (Table IV). Iron added to MPM cultures which had been mock-treated with MEM+BSA alone had no detectable consequence.

The latter protocol was used to find out also whether other

Table IV

Relative capacity of ferric, ferrous, zinc and cupric ions to restore the ability of ApoLF to enhance MPM killing of T. cruzi[a]

Macrophage pretreatment		No. of AMA per 100 MPM after	
Step A	Step B	2 hr	8 hr
MEM+BSA	MEM+BSA	23.3 ± 0.5	24.0 ± 4.5
LF 100% iron saturation	MEM+BSA	47.5 ± 1.3[b]	17.3 ± 4.5 [30.2] (63)
ApoLF	MEM+BSA	46.5 ± 1.7[b]	46.5 ± 6.0
ApoLF	12.5μM Fe^{+++}	45.6 ± 0.7[b]	20.0 ± 5.0 [25.6] (56)
ApoLF	12.5μM Fe^{++}	50.3 ± 6.6[b]	23.5 ± 3.5 [26.8] (53)
ApoLF	300 μM Zn^{++}	50.3 ± 4.0[b]	33.6 ± 3.7 [16.7] (33)
ApoLF	12 μM Cu^{++}	47.8 ± 1.5[b]	47.3 ± 2.7
MEM+BSA	12.5μM Fe^{+++}	24.5 ± 1.3	23.5 ± 1.3
MEM+BSA	12.5μM Fe^{++}	25.3 ± 2.3	23.8 ± 1.1
MEM+BSA	300 μM Zn^{++}	25.7 ± 1.0	23.8 ± 3.4
MEM+BSA	12.5μM Cu^{++}	23.8 ± 2.0	23.3 ± 3.7

For footnotes see next page

Footnotes to Table IV

[a]Step A: cultures of MPM were incubated with the solutions of the indicated materials at 37°C for 1 hr and washed with MEM. Step B: the cultures were further incubated with the indicated solutions at 37°C for 1 hr, washed and then incubated with AMA for 2 hr. Half of the cultures were terminated by fixation whereas the other half received MEM+dFBS and was incubated for an additional 6 hr. ApoLF or LF were used at 10 µg/ml.

[b]See footnote to Table I.

cations previously reported to bind LF (13, 14) could also restore the capacity of ApoLF to induce greater AMA killing. As shown in Table IV, ferrous but not cupric cations afforded total restoration, and zinc had only a partial effect.

To test whether the effects of LF on MPM interaction with T. cruzi AMA were characteristic of this protein or were shared by another iron-binding protein, parallel experiments were performed using iron-saturated LF, ApoLF, transferrin and apotransferrin. Neither transferrin, tested at concentrations ranging from 1 to 10,000 µg/ml, nor 1,000 µg apotransferrin per ml could enhance MPM association with or killing of T. cruzi whereas the effects of apoLF on MPM-AMA association and those of iron-saturated LF on both association and killing were readily seen (Table V). In related experiments we tried to restore the ability of ApoLF to enhance killing of T. cruzi AMA by pretreating the MPM with a 10µg/ml solution of this protein in the presence of 1000µg/ml iron saturated transferrin and later exposing these macrophages to the parasites. Whereas ApoLF, ApoLF in the presence of transferrin or transferrin itself were not able to stimulate killing of AMA by MPM, this ability was readily demonstrated by a solution of 100% iron saturated LF (data not shown).

Effects of scavengers of hydroxyl radical on AMA killing by LF-treated MPM. We have previously shown the involvement of at least H_2O_2, O_2^- and $^1O_2$ in the killing of T. cruzi AMA by LF-treated MPM (7). There have been reports that LF stimulates OH$^\cdot$ production by neutrophils (11) and iron ions have been shown to play a role in the formation of OH$^\cdot$ radicals via the Häber-Weiss reaction (15).

Table V

Lack of transferrin effect on the capacity of MPM to associate with or kill AMA[a]

Exp. No.	Macrophage pretreatment	No. of AMA per 100 MPM after 2 hr	8 hr
1	MEM+BSA	25.1 ± 2.0	26.3 ± 3.7
	ApoLF, 10µg/ml	51.3 ± 3.7	45.7 ± 6.0
	LF, 100% iron saturation, 10 µg/ml	50.6 ± 6.1	8.8 ± 1.2 [41.8] (83)
	Apotransferrin, 1,000 µg/ml	25.1 ± 4.5	22.3 ± 5.0
	Transferrin, 1,000 µg/ml	27.3 ± 0.5	26.0 ± 4.0
2	MEM+BSA	38.5 ± 2.2	39.1 ± 0.7
	Transferrin, 1 µg/ml	45.8 ± 4.0	38.0 ± 3.0
	Transferrin, 10 µg/ml	47.1 ± 2.7	45.8 ± 4.2
	Transferrin, 100 µg/ml	42.0 ± 6.1	42.5 ± 5.4
	Transferrin, 1,000 µg/ml	48.6 ± 5.4	43.0 ± 5.2
	Transferrin, 10,000 µg/ml	46.5 ± 3.5	42.8 ± 1.6

For footnotes please see next page

Footnotes to Table V

[a]Cultures of MPM were incubated with the indicated solutions at 37°C for 1 hr, washed with MEM and then incubated with AMA for 2 hr. Half of the cultures were terminated by fixation whereas the other half received MEM+dFBS and was incubated for an additional 6 hr. Transferrin was 100% iron saturated.

[b]See footnote to Table I.

Therefore, we looked into the possible involvement of $OH^{\cdot}$ in AMA killing in our assay system. The results indicated that thiourea and α-ketobutyric acid, both scavengers of $OH^{\cdot}$ radicals (12, 16), significantly reduced the extent of the enhancement of MPM cytotoxicity induced by LF (Table VI). Urea, a thiourea analog lacking this scavenging property, was ineffective. At the tested concentrations, thiourea, urea and α-ketobutyric acid did not affect the viability of MPM or the AMA to any appreciable extent (data not shown).

Table VI

Effects of scavengers of hydroxyl radicals on T. cruzi AMA killing by LF-treated MPM[a]

Macrophage pretreatment	No. of AMA per 100 MPM after 2 hr	8 hr
MEM+BSA	20.6 ± 2.9	20.3 ± 4.5
LF	42.3 ± 1.9[b]	14.3 ± 3.2 [38.0] (66)
LF + 50mM thiourea	38.8 ± 5.3[b]	34.7 ± 7.4
50mM thiourea	18.5 ± 2.2	15.1 ± 1.0
LF + 50mM urea	39.0 ± 4.4[b]	9.3 ± 2.8 [29.7] (76)
50mM urea	17.8 ± 1.2	15.3 ± 1.5
LF + 40mM α-ketobutyric acid	41.3 ± 1.2[b]	38.2 ± 2.0
40mM α-ketobutyric acid	17.1 ± 2.5	18.1 ± 2.7

For footnotes please see next page

Footnotes to Table VI

[a]When designed to be present, thiourea, urea or α-ketobutyric acid was added to the culture medium 2 hr after placing the MPM in the culture chambers and kept in it throughout the entire experiment, including washings. Otherwise, the protocol was as described in the footnote to Table I.

[b]See footnote to Table I.

DISCUSSION

The observation that iron-containing LF but not ApoLF enhanced the capacity of MPM to kill T. cruzi, together with the recovery of such activity afforded by iron restoration to ApoLF, contrasted with the lack of an iron requirement for this glycoprotein to enhance MPM-parasite association (i.e., binding and internalization). This difference infers at least two separate mechanisms for the noted stimulatory effects of LF. The increased capacity of MPM to take up AMA or latex beads after MPM incubation with either ApoLF or LF attributed this property to the glycoprotein itself. Since both ApoLF and LF were found to bind to the MPM surface and receptors for LF have been shown to be present on the surface of both MPM (17) and T. cruzi AMA (7), any of these molecules could have conceivably established a molecular bridge between MPM and AMA, facilitating parasite uptake in a manner similar to opsonization. However, ApoLF and LF also stimulated latex bead uptake by MPM, suggesting that a bridging mechanism was not necessary for MPM to denote a greater phagocytic capacity. Nevertheless, bridging can not be ruled out as a contributing mechanism.

The concept that enhanced AMA killing required the presence of iron in the LF molecule was supported by several lines of evidence. First, parasite killing was enhanced by iron-containing (whether at 20 or 100% saturation) LF but not by ApoLF. Second, enhanced killing was not seen when the MPM were treated with iron-containing LF in the presence of deferoxamine, an iron chelator. The magnitude of this effect was dependent upon the concentration of deferoxamine and comparable results

were obtained by using DTPA, another iron chelator. Third, we were able to induce killing enhancement with ApoLF after iron restoration. It is noteworthy that the tested concentrations of deferoxamine or DTPA were not cytotoxic for the MPM and, therefore, could not have prevented parasite disposal via killing the host cell.

The presence of DTPA in the culture medium during MPM treatment with LF prevented the enhancement of AMA killing. Since this chelator can not enter into living cells (12), these results suggest that DTPA must acquire iron from the LF molecule in the culture medium or while the protein is bound to the MPM surface to suppress greater killing activity. The idea that ApoLF is binding to the MPM surface is supported by the fact that the ability to enhance MPM killing of AMA was restored to ApoLF by free iron ions in the culture medium after ApoLF treatment of MPM (i.e., after the removal of non-bound protein).

Free iron ions did not induce greater parasite killing by MPM (Tables I and IV), indicating that this effect required the benefit of a carrier. Yet, a mere ability to bind and transport iron was not sufficient since the phenomenon could not be induced with transferrin (Table V).

Hypothetically, LF could provide distinct signals to MPM, leading to greater phagocytic and cytotoxic capacities. Binding of the glycoprotein, regardless of the presence of iron, would increase phagocytosis whereas iron would also be required to trigger the toxic mechanism(s). Alternatively, surface-bound LF might initiate the process that leads to increased killing but iron transported into cell by LF could be essential for the effector mechanism to be mounted. However, this possibility was not in keeping with the results of experiments in which the MPM were simultaneously treated with ApoLF and transferrin

[which is known to bind to surface receptors other than those for LF (17, 18) and should have transported iron into the cells], since killing was not increased under these conditions. On the other hand, iron brought in by transferrin might not have been found where ApoLF could have used it.

The present results do not specify if the presence of the appropriate cation in the LF molecule causes the molecule to bind to a specific MPM surface site(s) -different in some manner from that to which ApoLF binds- enabling it to elicit enhanced cytotoxicity. However, ApoLF did stimulate AMA killing when zinc was present instead of iron (Table IV). This finding could be interpreted in terms of a need for LF to be in a certain conformation (attained best with iron) necessary for the protein to bind to the proper receptor to be active in promoting killing or the participation of iron and/or zinc in the cytotoxic mechanism.

Iron plays a role in the generation of oxygen reduction metabolites (reviewed in reference 15), some of which have been shown to mediate MPM killing of T. cruzi AMA (7). Internalized LF might promote AMA killing within MPM via iron transport. If so, this property would be unique to LF because transferrin had no detectable effect on AMA killing by MPM even when used at 100% iron saturation and tested over a 10,000-fold concentration range (Table V). When either thiourea or α-ketobutyric acid, both scavengers of $OH^{\cdot}$, were present in the culture medium, the LF effect on parasite destruction was abrogated, suggesting that this oxygen reduction metabolite was involved in the effector mechanism. This observation adds $OH^{\cdot}$ to the list of oxygen metabolites that are produced by stimulated MPM and are directly or indirectly toxic for T. cruzi AMA [which until now included H_2O_2, $O_2^{\cdot-}$ and $^1O_2$ (7)],

and is in keeping with a previous report that LF stimulates $OH^{\cdot}$ production by neutrophils (11). In our earlier work, we found that sodium benzoate, a scavenger of $OH^{\cdot}$, would not inhibit the stimulatory effect of LF on AMA killing by MPM (7). The present results, showing that two other $OH^{\cdot}$ scavengers did inhibit such effect, implicated this reactive oxygen intermediate in AMA killing by LF-treated MPM but did not explain the negative results obtained with sodium benzoate.

We have found that LF levels are increased in the serum of acutely infected chagasic mice and that AMA from the spleens of these animals show intense fluorescence after incubation with fluorescein-labeled rabbit anti-LF (Lima and Kierszenbaum, unpublished results). This finding and the fact that LF presented to MPM on the surface of *T. cruzi* AMA also enhances phagocytosis and killing (7) indicate that the conditions for the production of the *in vitro* effects described in this paper are present in an infected host.

REFERENCES

1. **Masson, P. L., J. F. Heremans, and E. Schonne.** 1969. Lactoferrin, an iron-binding protein in neutrophilic leukocytes. J.Exp. Med.130:643.

2. **Spitznagel, J. K.** 1984. Nonoxidative antimicrobial reactions of leukocytes. In Regulation of Leukocyte Function. R. Snyderman, ed.Plenum Publishing Corp., New York. P. 283.

3. **Broxmeyer, H. E., A. Smithyman, R. R. Eger, P. A. Meyers, and M. de Sousa.** 1978. Identification of lactoferrin as the granulocyte-derived inhibitor of colony-stimulating activity production. J. Exp.Med. 148:1052.

4. **Broxmeyer, H. E., and E. Platzer.** 1984. Lactoferrin acts on I-A and I-E/C antigen subpopulations of mouse peritoneal macrophages in the absence of T lymphocytes and other cell types to inhibit production of granulocyte-macrophage colony stimulatory factors in vitro. J.Immunol. 133:306.

5. **Broxmeyer, H. E., L. Juliano, A. Waheed, and R. K. Shadduck.** 1985. Release from mouse macrophages of acidic isoferritins that suppress hematopoietic progenitor cells is induced by purified L cell colony stimulating factor and suppressed by human lactoferrin. J. Immunol.135:3224.

6. **Nishiya, K., and D. A. Horwitz.** 1982. Contrasting effects of lactoferrin on human lymphocyte and monocyte natural killer activity and antibody-dependent cell-mediated cytotoxicity. J. Immunol. 129:2519.

7. **Lima, M. F., and F. Kierszenbaum.** 1985. Lactoferrin effects on phagocytic cell function. I. Increased uptake and killing of and intracellular parasite by murine macrophages and human monocytes. J. Immunol. 134:4176.

8. **Aisen, P., and A. Leibman.** 1972. Lactoferrin and transferrin: a comparative study. Biochim. Biophys. Acta. 257:314.

9. **Bezkorovainy, A., and R. J. Zschocke.** 1974. Structure and function of transferrins. I. Physical, chemical, and iron-binding properties. Arzneim.-Forsch. 24:476.

10. **Villalta, F., and F. Kierszenbaum.** 1982. Growth of isolated amastigotes of Trypanosoma cruzi in cell-free medium. J. Protozool.29:570.

11. **Ambruso, D. R., and R. B. Johnston.** 1981. Lactoferrin enhances hydroxyl radical production by human neutrophils, neutrophil particulate fractions, and an enzymatic generating system. J. Clin. Invest. 67:352.

12. **Doroshow, J. H.** 1986. Role of hydrogen peroxide and hydroxyl radical formation in killing of Ehrlich tumor cells by anticancer quinones. Proc. Natl. Acad. Sci. USA 83:4514.

13. **Ainscough, E. W., A. M. Brodie, and J. E. Plowman.** 1982. Zinc transport by lactoferrin in human milk. Am. J.Clin. Nutr. 33:1314

14. **Masson, P. L., and J. F. Heremans.** 1968. Metal-combining properties of human lactoferrin (red milk protein). I. The involvement of bicarbonate in the reaction. Eur. J. Biochem. 6:579.

15. **Klebanoff, S. J.** 1982. Oxygen-dependent cytotoxic mechanisms of phagocytes. Adv. Host Defense Mech. 1:111.

16. **Starke, P. E., and J. L. Farber.** 1985. Ferric iron and superoxide ions are required for the killing of cultured hepatocytes by hydrogen peroxide. Evidence for the participation of hydroxyl radicals formed by an iron-catalyzed Haber-Weiss reaction. J. Biol. Chem. 260:10099.

17. **Van Snick, J. L., and P. L. Masson.** 1976. The binding of human lactoferrin to mouse peritoneal cells. J. Exp. Med. 144:1568.

18. **Bennett, R. M., and J. Davis.** 1981. Lactoferrin binding to human peripheral blood cells: an interaction with a β-enriched population of lymphocytes and a subpopulation of adherent mononuclear cells. J.Immunol. 127:1211.

APPENDIX

APPENDIX

Lactoferrin is a glycoprotein present in the neutrophil specific granules (1) and is normally found in plasma in trace amounts (2). Inflammatory conditions, such as bacterial infections (3,4) and arthritis (5), are accompanied by elevated concentrations of lactoferrin in plasma. Since inflammation with neutrophil infiltrates is present in acute Chagas' disease and lactoferrin modulates Trypanosoma cruzi uptake by macrophages and monocytes in vitro (6,7,8), we explored the possible presence of lactoferrin in infected mice and whether it bound the parasite in vivo.

In the first approach, acute and chronically infected mice were bled and their sera separated. An indirect immunofluorescence (IFI) test using cultured amastigote forms of T. cruzi (6) and fluorescein-conjugated anti-lactoferrin IgG was selected to detect binding of serum lactoferrin to the parasite. A second approach used spleens from infected mice as a source of amastigotes, testing for the presence of lactoferrin in situ by direct immunofluorescence using fluorescein-labeled anti-lactoferrin IgG.

Four week old Crl:CD-1(ICR)BR Swiss mice were purchased from Charles River Laboratories (Portage, MI). These mice were infected intraperitoneally (i.p.) with 1×10^5 blood trypomastigotes. The mice were bled at different times post-infection and their sera were pooled, aliquoted and stored at $20^{o}C$ until used.

The IFI was done as described previously (6) using culture amastigotes grown in ML-15H medium. The single cell suspensions used in the direct immunofluorescence studies were prepared in a Ten Broeck tissue grinder in phosphate buffered saline (PBS) from the spleens of mice sacrified on day 12 post-infection (p.i.). The suspensions were kept in an ice bath for one hour, and then used to prepare thin films on microscope slides, dried and fixed in methanol. The slides were then covered with normal rabbit IgG for 30 min, washed with PBS and incubated with FITC-labeled rabbit anti-lactoferrin IgG or FITC-labeled rabbit anti-human IgG as a control. After washing with PBS, the slides were examined by fluorescence microscopy.

As shown in Table 1, sera from acutely infected mice (collected on day 7 p.i., when no parasitemia was detectable and on day 15 p.i., when parasitemias attained peak levels) contained LF as detected on the surface of cultured amastigotes after incubation with sera and FITC-labeled anti-lactoferrin IgG. No fluorescence was detected when normal mouse serum or serum collected on day 49 p.i. were used.

Figure 1 shows splenic amastigotes displaying fluorescence after incubation with fluorescein-labeled anti-lactoferrin antibodies.

These observations revealed that lactoferrin was produced during the acute phase of the disease and reached levels detectable by our indirect immunofluorescence test, and that lactoferrin can bind amastigotes in their natural environment. Therefore, the conditions for lactoferrin binding to amastigotes released from infected cells are present *in vivo*. Whether these conditions lead to increased parasite uptake and killing by macrophages in the host remains a subject for further study.

TABLE I

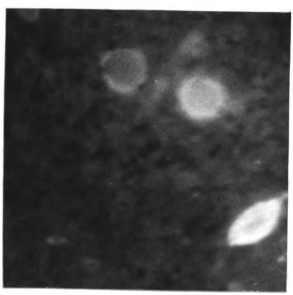
Increased Levels of LF in Acute Chagasic Sera

Amastigotes incubated with	Percent of LF-positive amastigotes (by IFI)
MEM - 1% BSA	0 ± 0.0 *
Normal mouse serum	0 ± 0.0
Infected mouse serum, day 7 p.i.	63.5 ± 3.5
Infected mouse serum, day 15 p.i.	70.4 ± 0.5
Infected mouse serum, day 49 p.i.	0 ± 0.0
10 ug/ml LF	58.4 ± 2.7

* Results are expressed as the Mean ± SD

Figure 1

Demonstration by direct immunofluorescence of lactoferrin binding to splenic amastigotes *in situ*. Parasites were obtained from spleens of infected mice at a time of peak parasitemia and stained by fluorescein-conjugated anti-lactoferrin IgG.



REFERENCES

1. Masson, P.L., Heremans, J.F., and Schone. 1969. Lactoferrin, an iron binding protein in neutrophilic leukocytes. J. Exp. Med. 130:643-658.

2. Birgens, H.S. 1985. Lactoferrin in plasma measured by an ELISA technique: evidence that plasma lactoferrin is an indicator of neutrophic turnover and bone marrow activity in acute leukemia. Scand. S. Herematol 34:326-331.

3. Hansen, N.E., Karle, H., Andersen, V., Malmquist, S., Hoff, G.E. 1976. Neutrophilic granulocytes in acute bacterial infection. Clin. Exp. Immunol. 26:463-468.

4. Gutteberg, T.J., Haneberg, B., Jorgensen, T. 1984. The latency of serum acute phase proteins in meningococcal septicemia, with special emphasis on lactoferrin. Clin. Chim. Acta 136:173-178.

5. Bennett, R.M., Skosey, J.L. 1977. Lactoferrin and lysozyme levels in synovial fluid: differential indices of articular inflammation and degradation. Arthritis Rheum 20:84-90.

6. Lima, M.F., and Kierszenbaum, F. 1985. Lactoferrin effects on phagocytic cell function I. Increased uptake and killing of intracellular parasite by murine macrophages and human monocytes. J. Immunol 134: 4176-4183.

7. Lima, M.F., and Kierszenbaum, F. 198 . Lactoferrin effects on the interaction of blood forms of Trypanosoma cruzi with mononuclear cells. Submitted.

8. Lima, M.F., and Kierszenbaum, F. 198 . Lactoferrin effects on phagocytic cell function II. Distinct roles in macrophage interaction with a parasite (Trypanosoma cruzi). Submitted.

SUMMARY AND CONCLUSIONS

A heavy inflammatory cell infiltrate consisting mainly of mononuclear cells, neutrophils and eosinophils is frequently seen in acute chagasic lesions. Secretion products of these cells may play a role in the interaction between Trypanosoma cruzi and the same or other inflammatory cells. In the course of this project, we focused on lactoferrin, a glycoprotein secreted by neutrophils, cells present in acute chagasic lesions. Levels of lactoferrin in the body fluids are known to be increased during inflammatory conditions such as found in acute Chagas' disease. This study examines the effects of lactoferrin on the phagocytosis of T. cruzi by macrophages and monocytes, which are also present in chagasic inflammatory infiltrates.

First, the modulatory effects of lactoferrin on the interaction between amastigotes or trypomastigotes with human monocytes or mouse peritoneal macrophages were analyzed. Lactoferrin was found to stimulate the uptake of amastigotes by either cell in a dose-dependent manner. This stimulatory effect was maximal at 10 µg/ml lactoferrin, a level comparable with that in individuals with inflammatory conditions. Higher concentrations of lactoferrin were found to be supraoptimal in that they did not effectively increase host-cell parasite association.

Pretreatment of either phagocytic cells or parasites with lactoferrin enhanced their association, suggesting that both macrophages and parasites bound the protein.

Reports by other investigators that macrophages and monocytes possessed receptors for lactoferrin were readily confirmed in this work and the presence of receptors for lactoferrin on amastigotes was demonstrated by binding and competition studies using ^{125}I-labeled lactoferrin. Since both parasites and phagocytes have receptors for this protein, lactoferrin might have bridged the two cells and thus increased their association. However, several lines of evidence suggested that parasite-cell bridging was not the sole explanation for this increase. First this enhancement was still demonstrated when lactoferrin-treated macrophages were incubated with α-methyl mannoside or anti-lactoferrin IgG (agents which block lactoferrin binding) before addition of the parasites. Second, lactoferrin enhanced trypomastigote-macrophage association despite the fact that these forms of the parasite do not bind lactoferrin. Finally, even the uptake of latex beads by macrophages was enhanced by treating these cells with lactoferrin. These findings suggest that lactoferrin has a nonspecific effect on macrophages which stimulates them to display a greater phagocytic activity, although bridging of parasites and cells may still contribute to this effect.

T. cruzi has been shown to be sensitive to the toxicity of the intermediates of oxygen metabolism whose production is stimulated during phagocytosis (reviewed on page 9). Lactoferrin has been shown to increase the production of some of these intermediates (reviewed on page 17). Therefore, the increased parasite destruction caused by lactoferrin could be due to the production of metabolites of the reduction of oxygen produced by phagocytes upon phagocytosis. Scavengers of $O_2^{\cdot-}$, H_2O_2, and $^1O_2$ were shown to abrogate the

lactoferrin-stimulated killing of amastigotes, and catalase, a scavenger of H_2O_2, inhibited trypomastigote killing. Therefore, lactoferrin may enhance T. cruzi destruction by macrophages via stimulation of the production of reactive oxygen intermediates.

Because lactoferrin is an iron-binding protein (reviewed on page 12) and iron has been implicated in the partial reduction of oxygen in phagocytic cells (reviewed on page 9), two separate approaches were used to investigate the contribution of iron to the lactoferrin effects. In the first, iron was removed from the protein by incubation with deferoxamine (an iron chelator). In the second approach, macrophages were pretreated with lactoferrin in the presence of deferoxamine and, after removal of both, exposed to amastigotes. In both cases, it was found that macrophages exposed to Fe-depleted lactoferrin were unable to kill the amastigotes. However, the cells retained their ability to phagocytize larger numbers of parasites than mock-treated controls.

The importance of iron in lactoferrin-mediated killing was confirmed by the restoration of this effect after incubation of apolactoferrin with ferric citrate. Lactoferrin preparations at 20 and 100% Fe-saturation were similarly active in stimulating parasite killing by macrophages.

The inability of Fe-depleted lactoferrin to stimulate parasite killing was unlikely to be due to a diminished ability of macrophages to bind the glycoprotein since all lactoferrin preparations, regardless of iron content, were found to bind these cells to the same extent.

The role of iron in the lactoferrin-stimulated killing of T. cruzi by macrophages was emphasized by the fact that while apolactoferrin, by itself, could not induce this effect, it could do so when its iron content was restored either before or after binding to the host cell. Interestingly, both ferric and ferrous ions were similarly effective. Addition of zinc instead of iron ions restored only 50% of the killing level, and cupric ions were totally ineffective.

The fact that Fe-saturated transferrin could not mimic the effects of lactoferrin argues against the possibility that lactoferrin stimulates killing of amastigotes by merely raising the intracellular iron content of the macrophages.

Next, we set out to explore the mechanism involved in the iron-dependent lactoferrin stimulation of parasite killing by macrophages. It should be borne in mind that our previous work had shown that this killing involved metabolites of the partial reduction of oxygen. Other investigations have reported that the production of $OH^{\bullet}$ and $^1O_2$ is dependent upon the presence of iron. The possible involvement of iron and these metabolites in parasite killing of lactoferrin-treated macrophages was studied by using iron chelators or scavengers of $OH^{\bullet}$ and $^1O_2$. Both were found to abrogate parasite killing to significant extents.

In summary, this research has shown that: a) lactoferrin can stimulate the phagocytic and cytotoxic properties of human monocytes and mouse peritoneal macrophages, b) some of the ensuing effects are dependent upon the presence of iron in the molecule, and c) reactive oxygen intermediates mediate the destruction of T. cruzi by

macrophages. Moreover, results described in the Appendix section point to the fact that lactoferrin is found in increased amounts in an *in vivo* infection with *T. cruzi* and that amastigotes bind to the protein *in situ*. These studies forward the notion that a product of an inflammatory cell modulates the function of another inflammatory cell type so as to increase the uptake and rate of killing of *T. cruzi* by the latter, and imply that lactoferrin may play a role in host defense against this parasite.

This research raises intriguing questions which deserve further attention. For example,

> What is the difference between amastigotes and trypomastigotes that enables the former but not the latter to express lactoferrin receptors and does this difference assume biological significance with respect to parasite survival and pathogenicity?
>
> What are the precise signals elicited in the macrophage upon binding of lactoferrin? What enables these cells to display greater cytotoxicity to *T. cruzi*?

The answers to these questions will contribute to the further understanding of the effects of lactoferrin in its modulation of macrophage - *T. cruzi* interaction, and also provide further insights into the macrophage activation pathways in general.